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62. THE ENZYMIC DECARBOXYLATION OF OXALOACETATE

By LESTER ORVILLE KRAMPITZ and CHESTER HAMLIN WERKMAN

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(*Received 21 April 1941*)

Although oxaloacetate has an important role in cellular metabolism, no enzyme has been described which brings about a rapid decarboxylation of the acid. Breusch [1939] investigated a heat-stable component of mammalian tissue responsible for this decarboxylation but could not identify it with any known physiological agent. The rate of this decarboxylation is comparatively slow. In investigations on the utilization of CO_2 by bacteria, Wood & Werkman [1938; 1940] postulated the reverse reaction, i.e. the carboxylation of pyruvate to form oxaloacetate. This reaction was represented as follows:

$$CO_2 + CH_3.CO.COOH \rightarrow HOOC.CH_2.CO.COOH.$$

Evans & Slotin [1940] have demonstrated the utilization of CO_2 with pigeon liver by the use of radioactive CO_2. The mechanism suggested by them is essentially the Wood & Werkman reaction. Krebs & Eggleston [1940], also working with pigeon liver, suggested that the reaction occurs in the dissimilation of pyruvate. They postulate that aneurin is required in the fixation. Smyth [1940] came to the same conclusion using vitamin-deficient staphylococci.

It is the purpose of the present communication to report the occurrence of a thermolabile enzyme which brings about the decarboxylation of oxaloacetate to pyruvate and CO_2. Experimental evidence is presented to show that (1) the magnesium ion and not cocarboxylase is a coenzyme for the enzyme; (2) the products of decarboxylation are pyruvate and CO_2; (3) the enzyme is heat-labile and (4) permeability of the cell wall is an important factor to be considered in studies on the enzyme. Furthermore, it is believed that under proper experimental conditions the enzyme described is capable of bringing about the carboxylation involved in CO_2 utilization.

Methods

Micrococcus lysodeikticus was employed in the investigation. The organisms were grown on a medium containing 1% glucose, 0·5% peptone, 0·3% yeast extract, 10% tap water and 2% agar. Inoculations were made on agar slopes in large Roux bottles. After 3 days' incubation at 30°, the cells were washed from the agar surface with distilled water, filtered through glass wool and washed twice in a centrifuge.

Acetone-treated cells. 10 g. of cell paste were mixed with 70 ml. ice-cold acetone and shaken thoroughly for 5 min. The cells were filtered off, suspended in 140 ml. ice-cold acetone and shaken vigorously for 10 min. This process was repeated. The preparation was then placed *in vacuo* to remove traces of acetone. These cells are permeable to oxaloacetate, whereas untreated cells are not.

Lysed cell preparation. To a 10% cell suspension (wet weight basis), one-tenth volume of saliva was added, and the mixture incubated 1 hr. at 36°, whereupon lysis occurred. For control, a 10% cell suspension without saliva was incubated in like manner.

Cocarboxylase- and Mg^{++}-deficient acetone-treated cells. The usual alkaline phosphate washing was employed. 1 g. of acetone-treated cells was washed twice for 10 min. on the shaking machine with 50 ml. phosphate buffer, *p*H 8·0, and once with 50 ml. distilled water. The cells maintain their deficiency if dried on a porous plate. Cocarboxylase and Mg^{++} cannot be removed by phosphate washing unless the cells are first treated with acetone.

Cocarboxylase- and Mg^{++}-deficient preparation of lysed cells. To 4 ml. of a 20% solution of lysed cells, 6 ml. of saturated $(NH_4)_2SO_4$ were added. The mixture was vigorously shaken for 10 min. and centrifuged. The precipitated particles were made up to a volume of 4 ml. with distilled water and the $(NH_4)_2SO_4$ washing was repeated twice. Finally, the precipitate was washed with distilled water and made up to the original volume of 4 ml.

Preparation of oxaloacetic acid. To the Na salt of diethyloxaloacetate (100 g. in 250 ml. water) conc. H_2SO_4, equivalent to the Na, was added. The free ester was removed as an oily layer by means of a separating funnel and dissolved in 80 ml. ether. The ether solution was washed twice with 50 ml. distilled water, and the ether evaporated off. The ester was hydrolysed by treatment with 4 vol. conc. HCl at room temperature and vigorously shaking the acid solution mechanically for at least an hour. The solution was then placed at −26° for 24–48 hr. The precipitated acid was removed by filtration, dissolved in the requisite amount of warm acetone and recrystallized by adding chloroform. The purity of such a sample as determined by the aniline citrate method [Ostern, 1933] is greater than 98%.

Pyruvic acid was determined by the carboxylase method [Krebs *et al.* 1940] and also by the salicylaldehyde method of Straub [1936]. CO_2 was determined by adding 0·3 ml. of 1 : 1 H_2SO_4 to the reactants in the manometer vessel.

The Barcroft-Warburg respirometer was used in this study. The total volume of reactants in each cup was 2 ml. Experiments conducted with untreated cells showed an endogenous activity which was deducted from values obtained with the substrates present. Endogenous activity of acetone-treated cells was negligible.

Experimental

Permeability. The untreated cells of *M. lysodeikticus* aerobically oxidized pyruvate, oxaloacetate, malate, fumarate and succinate with almost equal rapidity (Table 1). Anaerobically there was no evolution of CO_2 from any of these salts. The acetone-treated cells behaved similarly to the untreated cells excepting that they oxidized succinate very slowly and decarboxylated oxaloacetate very rapidly anaerobically. Since there was no increase in CO_2 evolution in the presence of the untreated cells and oxaloacetate, it may be assumed that the oxaloacetate did not reach the active enzyme centres. Apparently, the acetone extraction of the cells makes the cell permeable to oxaloacetate. Objections to this explanation may be made, inasmuch as the untreated cells showed an O_2-uptake with oxaloacetate. It is probable, however, that the oxaloacetate did not penetrate the untreated cell even in the aerobic experiments, and that the O_2-uptake was due to the oxidation of pyruvate formed by the spontaneous decarboxylation of oxaloacetate. This explanation is supported by the fact that anaerobically with the acetone-treated cells, the quantity of

Table 1. *Anaerobic and aerobic behaviours of* Micrococcus lysodeikticus *on the C_4 dicarboxylic acids and pyruvate*

	Untreated cells		Acetone-treated cells		
Substrate	Aerobic μl. O_2 uptake	Anaerobic μl. CO_2 evolved	Aerobic μl. O_2 uptake	Aerobic μl. CO_2 evolved	Anaerobic μl. CO_2 evolved
Pyruvate	248	6	262	320	12
*Oxaloacetate	225	4	255	1220	915
Malate	250	2	298	—	3
Fumarate	228	3	314	—	2
Succinate	240	1	12	—	0

Total volume of reactants = 2 ml. Conc. of substrates 0·025 *M*, 20 mg. cells per cup (dry weight), 0·125 *M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* All values corrected for spontaneous decarboxylation of oxaloacetate. Endogenous values deducted in experiments containing substrates.

CO_2 from oxaloacetate (915 μl.) is substantially equivalent to the CO_2 from the corresponding aerobic experiment (1220 μl.) minus the CO_2 from the aerobic pyruvate experiment (320 μl.). Apparently the excess CO_2 in the aerobic experiment is the result of pyruvate dissimilation. Pyruvate is not dissimilated anaerobically; consequently the CO_2 evolved from oxaloacetate under these conditions must have arisen by its decarboxylation, shown by the 915 μl. of CO_2 obtained anaerobically from 928 μl. of oxaloacetate.

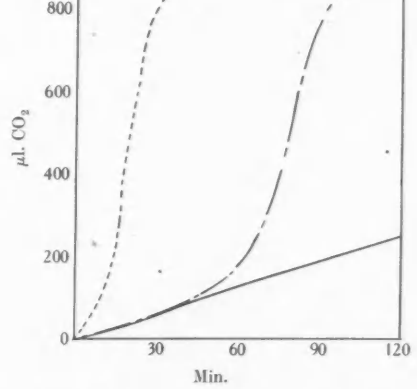

Fig. 1. Effect of lysis on the decarboxylation of oxaloacetate. Total volume of reactants 2·0 ml. Anaerobic; 0·125 *M* PO_4 buffer *p*H 6·8, temp. 30°. —— 2·5 % untreated cells + 920 μl. oxaloacetate. - - - - 2·5 % lysed cells + 920 μl. oxaloacetate. —·— 2·5 % untreated cells + 0·1 volume saliva + 920 μl. oxaloacetate.

Objections may also be raised that if the other C_4 acids reach their enzyme centres, oxaloacetic acid should likewise do so. Apparently other compounds having the grouping —CO.CH_2.CO— do not penetrate the cell wall, e.g. malonic acid, generally recognized as an inhibitor of succinate oxidation, shows no

inhibition with *M. lysodeikticus*, even after long incubation before the succinate addition. Apparently, the malonate does not penetrate the cell to the enzyme centre. This hypothesis cannot be tested with our acetone preparation, inasmuch as it does not oxidize succinate (Table 1).

Fig. 1 further substantiates the cell permeability hypothesis. *M. lysodeikticus*, as shown by Fleming [1924], is lysed by lysozyme, an enzyme present in both egg albumin and saliva. In the figure the decarboxylations of oxaloacetate by untreated cells, by previously lysed cells and by cells that were lysed during the course of the experiment are compared. If cell permeability is limiting the decarboxylation of oxaloacetate, it is to be expected that as lysis proceeds the enzyme will become liberated and be available for decarboxylation. Decarboxylation by the untreated cell was identical with the spontaneous decarboxylation; therefore the two curves are superimposable. The lysed preparation showed a remarkable ability to decarboxylate oxaloacetate, and the experiment in which lysis proceeded in the presence of oxaloacetate showed an increasing rate of decarboxylation. Saliva alone does not increase the spontaneous decarboxylation. The results of these experiments clearly establish the importance of cell permeability.

Products of the decarboxylation of oxaloacetate. Pyruvic acid and CO_2 are formed by the anaerobic breakdown of oxaloacetate (Table 2). For each oxaloacetic acid molecule decarboxylated, one molecule of pyruvate and one of CO_2

Table 2. *Products of anaerobic decarboxylation of oxaloacetate by an acetone preparation*

Material determined	Method of determination	Quantity obtained (μl.)	Recovery %
Oxaloacetate dissimilated	Aniline citrate	998	—
Pyruvate	Carboxylase	958	97
Pyruvate	Salicylaldehyde	970	98
CO_2	Acidification	947	96

were found. The pyruvate determinations by both the carboxylase and the salicylaldehyde methods agreed, and there was an equivalent recovery of CO_2 on acidification. The oxaloacetate was determined by the aniline citrate method.

Effects of Mg^{++} and cocarboxylase on the enzyme. The question next arises as to the relationship between oxaloacetate carboxylase and pyruvate carboxylase. Krebs & Eggleston [1940] and Smyth [1940] are of the opinion that the two are either identical or very similar. It is well known that yeast carboxylase not only decarboxylates pyruvate but also oxaloacetate. The products of decarboxylation of the latter are acetaldehyde and two molecules of CO_2. There are two possible mechanisms by which yeast carboxylase may decarboxylate oxaloacetate: initial β-decarboxylation or initial α-decarboxylation.

β-decarboxylation

(1) $HOOC.CH_2.CO.COOH \rightarrow CH_3.CO.COOH + CO_2$
$CH_3.CO.COOH \rightarrow CH_3.CHO + CO_2$

α-decarboxylation

(2) $HOOC.CH_2.CO.COOH \rightarrow HOOC.CH_2.CHO + CO_2$
$HOOC.CH_2.CHO \rightarrow CH_3.CHO + CO_2$ (spontaneous).

To obtain information relative to this question, the Mg^{++} and the cocarboxylase requirements of the two reactions have been determined. It is well known that

in both the aerobic and the anaerobic decarboxylations of pyruvic acid, cocarboxylase and Mg^{++} are required. With *M. lysodeikticus* it was found that the organism likewise requires cocarboxylase and Mg^{++} for the α-decarboxylation. On the contrary, for the β-decarboxylation of oxaloacetate only Mg^{++} is required. The difference in the coenzyme requirements indicates that the two enzymes are not alike. Whether decarboxylation of oxaloacetate by yeast involves two enzymes or occurs solely by α-decarboxylation must await further investigation. The present investigation shows that with bacteria the former is the case.

Data in Table 3 show the results with acetone-treated cells made deficient in cocarboxylase and Mg^{++}. Deficiencies were determined by observing the lack of ability of the preparation to oxidize pyruvate and oxaloacetate aerobically.

Table 3. *Pyruvate and oxaloacetate metabolism by Mg^{++} and cocarboxylase deficient and non-deficient acetone-treated cells*

	Deficient cells plus						Non-deficient acetone-treated cells
Substrate	No addition	Mg^{++}	Cocarboxylase	Mg^{++} and cocarboxylase	Mn^{++}	Mg^{++} and thiamin	
Pyruvate, μl. O_2 uptake	14	16	17	212	18	18	250
*Oxaloacetate, aerobic, μl. O_2 uptake	18	20	19	219	22	22	255
*Oxaloacetate, aerobic μl. CO_2 formed	41	927	38	1206	932	926	1228
*Oxaloacetate, anaerobic, μl. CO_2 formed	32	930	30	930	934	926	928

Total volume of reactants 2 ml. 0·025 *M* oxaloacetate, 0·006 *M* $MgCl_2$, 0·006 *M* $MnSO_4$, 25 μg. cocarboxylase or thiamin, 20 mg. cells per cup (dry weight), 0·125 *M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values of oxaloacetate deducted from substrate values. With Mg^{++} 178 μl. Without Mg^{++} 94 μl.

Upon addition of Mg^{++} and cocarboxylase to the deficient cells, the oxidation of pyruvate took place. 212 μl. O_2 were taken up with cocarboxylase and Mg^{++} addition, and only 14 μl. with no addition. The non-deficient acetone-treated cells took up 250 μl. O_2. When either Mg^{++} or cocarboxylase alone was added, no oxidation occurred. Therefore, it is apparent that both Mg^{++} and cocarboxylase are essential for pyruvic acid oxidation. The addition of aneurin and Mg^{++} had no effect, indicating that the mechanism for phosphorylating the vitamin had been destroyed by the acetone treatment.

The difference between pyruvate and oxaloacetate decarboxylations is apparent if the effects of Mg^{++} and cocarboxylase on oxaloacetate dissimilation are considered. Considering O_2 uptake alone, the difference is not evident, since both Mg^{++} and cocarboxylase are required for O_2 utilization. This oxidation does not involve oxaloacetate but pyruvate arising from the decarboxylation of the oxaloacetate. The picture, however, is different with respect to CO_2 evolution. The deficient cells were unable to decarboxylate oxaloacetate, e.g. only 41 μl. CO_2 were liberated. The addition of Mg^{++} restored both the anaerobic decarboxylation (930 μl.) and the aerobic decarboxylation (927 μl.) to the original values, which were comparable with those found when both Mg^{++} and cocarboxylase were added (930 μl. anaerobically and 1206 μl. anaerobically). Obviously cocarboxylase is not required for the decarboxylation of oxaloacetate. Mg^{++} increased the spontaneous decarboxylation of oxaloacetate and proper corrections were made.

In Table 4 data are given which were obtained with a lysed deficient preparation. With this preparation it should be stated that the dehydrogenase activity had been almost destroyed by lysing; consequently there was little O_2 uptake with pyruvate. This is in accordance with the findings of Penrose & Quastel [1930]. Values representing this O_2 uptake, therefore, have been omitted from the Table. The oxaloacetate decarboxylation values, however, show substantially the same results as those obtained with the acetone-treated preparation.

Table 4. *Metabolism of deficient and non-deficient lysed cells with oxaloacetate*

	Deficient lysed cells plus						
Substrate	No addition	Mg^{++}	Cocarboxylase	Mg^{++} and cocarboxylase	Mn^{++}	Mg^{++} and aneurin	Non-deficient lysed cells
*Oxaloacetate, anaerobic, μl. CO_2	52	928	48	934	927	922	917
*Oxaloacetate, aerobic, μl. CO_2	54	925	49	916	922	918	920

Total volume of reactants 2 ml. 0·025 *M* oxaloacetate, 0·006 *M* $MgCl_2$, 0·006 *M* $MnSO_4$, 25 μg. cocarboxylase or aneurin, 20 mg. cells per cup (dry weight), 0·125 *M* PO_4 buffer *p*H 6·8; aerobic exp. contained air in gas space, anaerobic contained N_2. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation of oxaloacetate values deducted. With Mg^{++} 178 μl. Without Mg^{++} 94 μl.

Effect of heat on the enzyme. It is well known that there is a heat-stable component present in mammalian tissue that activates the decarboxylation of oxaloacetate [Breusch, 1939]. The heat stability of our enzyme therefore has been studied.

That our preparation is not the heat-stable component is shown by Table 5. These results indicate that the enzyme is heat-labile and is almost completely destroyed at 100° in 5 min. The small value obtained under these conditions may be due to the same component discussed by Breusch.

Table 5. *Effect of heat on enzyme*

	Lysed preparation				Acetone preparation	
Substrate	No heat	60° 10 min.	80° 10 min.	100° 5 min.	No heat	100° 5 min.
*Oxaloacetate, anaerobic, μl. CO_2	944	498	40	15	948	30

0·025 *M* oxaloacetate, 0·125 *M* PO_4 buffer *p*H 6·8, 2·5% lysed preparation per cup; 20 mg. acetone preparation per cup. Gas space N_2. Total volume of reactants 2·0 ml. Temp. 30°. Time 1 hr.

* Spontaneous decarboxylation values deducted.

Discussion

The results reported here appear to differ from those reported by Smyth [1940] and Krebs & Eggleston [1940]. These investigators believe that aneurin is necessary for the synthesis of oxaloacetate from pyruvate and CO_2. Smyth, working with staphylococci, showed that oxaloacetate could replace aneurin in the dismutation of pyruvate. He concluded that aneurin is required for the synthesis of oxaloacetate, which acts as a hydrogen acceptor in the dismutation

of pyruvate to lactate, acetate and CO_2. Therefore in the presence of oxaloacetate the vitamin is not necessary for the dismutation. If this were the case, our vitamin-deficient acetone preparation should oxidize pyruvate in the presence of oxaloacetate. This was not true in our experiments, since O_2-uptake and decarboxylation of pyruvate are obtained only on addition of Mg^{++} and cocarboxylase to the oxaloacetate experiments. It must be borne in mind that with oxaloacetate as the substrate, there is a sufficient supply of pyruvate at all times, owing to spontaneous decarboxylation of oxaloacetate. It should be pointed out that the results of Smyth are by no means conclusive proof of the claimed function of the vitamin. Although the anaerobic dissimilation of pyruvate has not been studied as thoroughly as the aerobic oxidation, it is probable that the component parts of the system are very similar. In the aerobic dissimilation of pyruvate [Lipmann, 1939; Banga *et al.* 1939], cocarboxylase, a C_4 dicarboxylic acid, inorganic phosphate, adenine nucleotide and Mg^{++} are components of the system. The oxidation thus requires cocarboxylase even in the presence of C_4 acids. Certainly cocarboxylase, in this case, has a function other than in the synthesis of C_4 acids. The system investigated by Smyth is very complex and there is a probability of a multiple deficiency. The removal of any one of these deficiencies might stimulate the reaction. The speed of the reaction may be limited by an additional factor when either aneurin or C_4 acids or both are added. Particularly in view of our findings, it seems that there may be another explanation of Smyth's results.

Krebs & Eggleston [1940] investigated the dissimilation of pyruvate by pigeon liver and found an increased pyruvate disappearance in the presence of $NaHCO_3$ and CO_2. They concluded that the pyruvate was used in CO_2 fixation, forming oxaloacetate which then undergoes reactions of the citric acid cycle. Aneurin was concluded to be concerned with the fixation reaction because the vitamin stimulated pyruvate dissimilation by liver from an avitaminous pigeon. This stimulation was not obtained by employing breast muscle tissue from the same bird. They concluded that the vitamin acts in a reaction occurring in the dissimilation by liver but not in that by breast muscle. Furthermore, since oxaloacetate synthesis was considered to occur in liver but not in breast muscle, the vitamin was thought to act in this synthesis. The suggested function of the CO_2 is in accord with the observations made with bacteria and is attractive particularly in view of the results of Evans & Slotin [1940]. The evidence for the function of the vitamin in this reaction is, however, not conclusive. Krebs & Eggleston presented no proof that the breast muscle was deficient in aneurin. It cannot be assumed that in an animal suffering from vitamin deficiency all of the organs are equally deficient [cf. Ochoa & Peters, 1938]. Thus the liver of an avitaminous pigeon may be limited in its metabolism of pyruvate, whereas the breast muscle may still contain sufficient cocarboxylase for an active reaction. Therefore, the evidence offered by Krebs & Eggleston that aneurin does not function in pyruvate metabolism of breast muscle needs confirmation, since the vitamin activity will not be apparent if the muscle is not deficient, and therefore their conclusion concerning the function of the vitamin in oxaloacetate synthesis may have another explanation.

We have been unable by use of the enzyme from *M. lysodeikticus* to obtain carboxylation of pyruvate. Inasmuch as the ΔF of the fixation reaction is positive, and therefore requires energy from a coupled reaction, the vitamin may be required in this second reaction. The possibility exists that in our preparation conditions were such that the enzyme catalysing the energy-yielding reaction has been destroyed, and consequently we obtain only the decarboxylation

reaction. We are at present attempting to reverse the reaction to obtain carboxylation. It is to be remembered that our preparation was inactive with aneurin but active with the phosphorylated vitamin, indicating that the phosphorylating mechanism has been destroyed. It is probable that the energy-yielding reaction necessary for carboxylation may be coupled with a phosphate exchange which did not occur in our preparation.

Summary and conclusions

An enzyme preparation obtained from *Micrococcus lysodeikticus* is described which catalyses the reaction: $HOOC.CH_2.CO.COOH \rightarrow CH_3.CO.COOH + CO_2$. Magnesium ions are apparently required for the reaction, whereas cocarboxylase and aneurin have no effect. The enzyme is heat-labile, and thus is not the heat-stable compound occurring in mammalian tissue which stimulates the decarboxylation of oxaloacetate. Indications are that the enzyme is required in the carboxylation reaction involved in the CO_2 utilization of Wood & Werkman.

Permeability relationships must be considered in working with oxaloacetate and whole cells of *Micrococcus lysodeikticus*.

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63. PROLIFERATION-PROMOTING INTER-CELLULAR HORMONES

2. EVIDENCE FOR THEIR PRODUCTION BY LIVING CELLS AS A RESPONSE TO INJURY

By JOHN R. LOOFBOUROW,[1] SISTER CECELIA MARIE DWYER, S.C., and SISTER ANN GERTRUDE CRONIN, S.C.

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(*Received 19 May 1941*)

Previously published investigations from these laboratories indicate that when yeast, various other micro-organisms, living tissues of rat, mouse and chicken embryos, and living adult newt tissues are subjected to such varied forms of injury as lethal ultraviolet radiation, X-rays, mechanical injury, chemical irritation and oxygen lack, there appear in the intercellular fluids factors stimulating cellular proliferation [Fardon *et al.* 1937; Norris & Ruddy, 1937; Sperti *et al.* 1937, 1, 2; Loofbourow *et al.* 1938; 1939; 1940]. We have called these factors 'proliferation-promoting intercellular hormones'.[2] Davidson [1940] has recently confirmed our results in the case of proliferation-promoters induced by the irradiation of yeast with lethal ultraviolet.

The possibilities that intercellular hormones from injured cells may act as the initial stimulus in wound healing, and that they may play a part in over-growth and neoplasia, are obvious reasons for interest in this problem. The conception of a relationship between the products of injured tissues and the repair processes following injury is not new. There is, in fact, an extensive literature regarding this question [for example, see Wiesmer, 1892; Carrel, 1930; Arey, 1936; Menkin, 1940; Loofbourow, 1941]. The techniques employed by our colleagues and ourselves [Sperti *et al.* 1937, 1, 2, 3; Loofbourow *et al.* 1938; 1939; 1940] make possible the controlled comparison of the proliferation-promoting effects of products from injured and uninjured tissues, and hence afford a new quantitative approach to the problem.

In investigating this problem, it seems of importance to distinguish between (*a*) factors released as a result of cell disintegration; (*b*) factors, already in the cell, released as a result of increased membrane permeability; and (*c*) factors synthesized by living, injured cells and released by them into the intercellular fluids. *A priori*, it is conceivable that the proliferation-stimulating activity found in the intercellular fluids of suspensions of injured cells might be attributable entirely to (*a*) or to (*b*) and (*a*). As will appear from the following, however, the experimental evidence seems to indicate that factors of the (*c*) type play a major part in the phenomenon.

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[2] The term 'intercellular hormone' was suggested by Loofbourow & Morgan [1938] for factors from cells which act through the intermediary of the intercellular fluids in affecting the metabolism of the cell community.

Experimental methods and results

In the following experiments, yeast (*S. cerevisiae*, Fleischmann Bakers' strain) was both the organism injured and the test organism for determining the proliferation-promoting activity of the intercellular fluids. Staining with methylene blue was used to estimate the number of cells killed [Richards, 1932] in the suspensions subjected to injury.

The technique of the growth tests has been described in detail elsewhere [Loofbourow, Dwyer & Morgan, 1938]. The potencies are evaluated in terms of growth units per mg. of dry material or per ml. of fluid, one growth unit being the amount of material (mg. or ml.) which must be added to 1 ml. of culture to cause an increased 24-hr. yeast crop of 1·6 mg./ml. (wet weight) as compared with the controls, under the standard conditions adopted for the growth tests. Since the control crop is normally about 0·16 mg./ml., one growth unit is also (approximately) the quantity of material which must be added to each ml. of culture medium to increase the 24-hr. crop by 1000%.

1. *Effect of rapid killing compared with that of slow injury.* If proliferation-promoting factors arise in the intercellular fluids primarily as a result of mechanism *a* (disintegration of dead cells), one would expect yields to be nearly the same from the same number of dead cells, whether they were killed rapidly or slowly. If they arise as a result of *b* (increased permeability) or *c* (production of factors by living injured cells), greater yields might be expected from cells killed slowly.

To test this point, yeast was suspended in isotonic salt solution at a concentration of 20 g./l. Aliquots were irradiated with full ultraviolet from a quartz mercury arc for times of 20, 40, 80, 160 and 320 min. The intensities were adjusted (by varying the distances of the samples from the arc) so that approximately 90% of the cells were dead in each aliquot at the end of its irradiation period. Corresponding control aliquots stood without irradiation for the same lengths of time. Immediately following each irradiation period, the irradiated suspension and the control suspension corresponding to it were filtered through Berkefeld filters, and the cell-free filtrates were taken to dryness. The residues of cells were discarded. For growth tests, the dried products were made up in distilled water to one-fifth their original volume, and assayed by the usual techniques (loc. cit.).

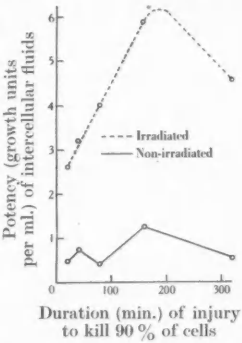

Fig. 1. Effect of decreased rate of killing on increased yield of proliferation-promoting factors.

The results of such an experiment are shown in Fig. 1. In the case of irradiated suspensions, the potency of the extract was approximately a linear function of the duration of injury up to 160 min., after which there was a marked decrease, the cause of which has not been established. Since the same numbers of cells were killed in all instances, these results indicate that the production of active factors was probably not primarily by mechanism *a* (disintegration of dead cells). In these particular experiments, the longer irradiation periods corresponded, also, to longer extraction periods. Hence either mechanism *b* or mechanism *c* could account for the results.

2. *Relation of yield to number of cells killed.* In a series of seven experiments, yeast suspended in isotonic salt solution was irradiated with full ultraviolet at

intensity levels which varied from experiment to experiment. Aliquots were removed at intervals for the determination of percentages of dead cells by methylene blue staining, and of the potencies of the intercellular fluids. Two examples, which are representative, are summarized in Fig. 2. The maximum staining referred to in each instance was between 97 and 100 %.

It will be noted from these examples that from 15 to 20 % of the maximum potency was attained by the time 2 % of the cells were killed, and roughly one-third of the maximum potency by the time 10 % of the cells were killed. This

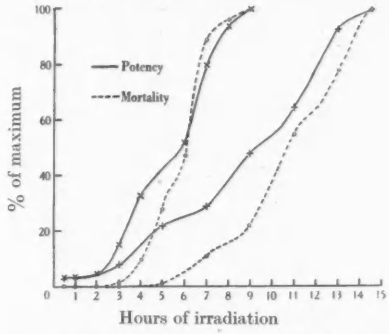

Fig. 2. Relation of yield of proliferation-promoting factors to mortality.

rather rapid rise in potency during the early periods of irradiation, before appreciable killing took place, seems unattributable to mechanism *a* and seems more easily explained by mechanism *b* or *c*.

3. *Effect of injuring cells in various suspension media.* Cells suspended in distilled water or isotonic salt solution have no source of building materials for the synthesis of proliferation-promoting factors other than their own protoplasm. Consequently, if mechanism *c* (production of active materials by living, injured cells) accounts in part for the phenomena observed, one might expect to obtain greater yields of active materials from cells suspended in media containing available sources of nitrogen, phosphorus, carbohydrate etc.

Yeast was suspended at a concentration of 100 g. (wet weight) per l. in the following media: distilled water, isotonic NaCl, Reader's medium[1] [Reader, 1927], 95 % alcohol, and Ringer's glucose phosphate solution.[2] These suspensions were irradiated simultaneously in quartz test tubes with full ultraviolet at a distance of 15 cm. from a quartz mercury arc until 100 % of the cells in the salt solution stained when tested with methylene blue. Under these conditions, practically complete killing was obtained in all suspensions. Similar suspensions were kept standing without irradiation during the same interval as controls. All suspensions were then filtered through Berkefeld filters, and the cell-free fluids were taken to dryness. The dried products were dissolved in distilled water to one-fifth their original volume, taking care to suspend all the material. These solutions were assayed in the usual way. Table 1 shows the results.

[1] The composition of Reader's medium per 100 ml. is as follows: $(NH_4)_2SO_4$, 0·3 g.; $MgSO_4$, 7HO, 0·07 g.; KH_2PO_4, 0·10 g.; K_2HPO_4, 0·016 g.; NaCl, 0·05 g.; $Ca(NO_3)_2$, 0·04 g.; glucose, 0·5 g.

[2] The composition per 100 ml. was as follows: NaCl, 0·09 g.; $CaCl_2$, 0·024 g.; KCl, 0·001 g.; $Na_2H_2P_2O_7$, $6H_2O$, 0·006 g.; $NaHPO_3$, $3H_2O$, 0·205 g.; glucose, 0·02 g.

It will be noted that the most highly potent irradiation product and the least potent non-irradiated extract were those from cells suspended in Reader's medium. In the case of alcohol, the combined lethal effects of alcohol and irradiation resulted in lower potency per ml. of filtrate than extraction with alcohol alone. Since alcohol is an effective solvent for extracting growth-promoters of the bios type [e.g. Narayanan, 1930], it is not surprising that the alcohol preparations showed high activity.

All cells were killed in the various irradiated suspensions. Hence it is difficult to attribute the observed results to mechanism *a* alone. If increased permeability accounted entirely for the difference in potency of the irradiated and non-irradiated extracts, why was the difference greatest in the case of Reader's medium? If, on the other hand, mechanism *c* played a part in the phenomenon, the increased effect in Reader's medium is understandable because of the carbohydrate, nitrogen, phosphorus etc. supplied by it to the cells.

Table 1. *Effect of the suspension medium on the yield of proliferation-promoting factors from injured cells*

Suspension medium	Potency G.U. per ml.	
	Not irrad.	Irrad.
Distilled water	2·95	11·8
Isotonic NaCl	2·17	9·52
95% alcohol	13·3	10·9
Reader's medium	0·77	15·4
Ringer's G.P. solution	2·67	14·3

4. *Effects of CO_2 and O_2.* Because of the fermentation-stimulating effect of filtrates from irradiated as compared with non-irradiated cells [Cook & Loofbourow, unpublished], it was believed of interest to determine the effects of CO_2 and O_2 on the production of proliferation-promoting factors by yeast [Loofbourow & Dwyer, 1939]. In six experiments, yeast, at a concentration (wet weight) of 100 or 200 g./l., was suspended in isotonic NaCl. The suspensions were divided into three portions of 75 ml. each. Through one portion, CO_2 was bubbled continuously; through another portion O_2. The third portion had nothing bubbled through it. After 24 or 48 hr. of such treatment, the suspensions were filtered through Berkefeld filters, and the cell-free fluids were dried and assayed.

In these experiments, the cells were not killed. The maximum staining observed at the ends of the periods was of the order of 2%. The greatest potencies were in the filtrates from yeast exposed to CO_2, the next in the control filtrates, and the least in the filtrates from the cells exposed to O_2. Table 2 is from a

Table 2. *Effect of CO_2 on the production of proliferation-promoting factors by yeast*

Sample	Cell-free filtrates Potency G.U. per ml.
CO_2	6·6
Untreated	3·1
O_2	1·5

representative experiment. Since no appreciable killing took place in these experiments, mechanism *a* appears improbable, and mechanisms *b* and *c* more likely. The effect of CO_2 on membrane permeability is well known. Hence one cannot say from these experiments whether *b* or *c* or both were involved.

The possible effect of accumulated CO_2 in leading to production of proliferation-promoting factors must evidently be considered when investigating the action of various injuring agents in inducing the production of such factors.

5. *Comparison of yields from cells fragmented by grinding before and after irradiation with lethal ultraviolet.* The following experiments were suggested by Prof. A. J. Carlson of the University of Chicago. They are based on the consideration that if active factors are produced by living cells as a response to injury (mechanism *c*), the yield should be less from cells ground until they are fragmented and then irradiated dead than from cells irradiated while alive and then ground. In both instances the end-products would, theoretically, be the total water-soluble cell contents freed from the cell—hence no questions of membrane permeability or efficiency of extraction should be involved.

Two equal weights of moist yeast (10–30 g.) were taken. One of these was ground with sand in a mortar until the cells appeared to be well fragmented. Both portions were suspended in isotonic NaCl at a concentration of 100 g./l. and irradiated in quartz test tubes with full ultraviolet until 50–100 % (the percentage varied from experiment to experiment) of the cells in the unground suspension were killed. The suspensions were centrifuged, and the supernatant fluids decanted. The unground cells were then ground in a mortar, after which both residues were resuspended in their respective fluids. The suspensions were later filtered through Berkefeld filters, and the cell-free filtrates dried and assayed. The purposes of filtration were to remove insoluble residues which might interfere with the optical estimation of yeast growth and to render the solutions sterile.

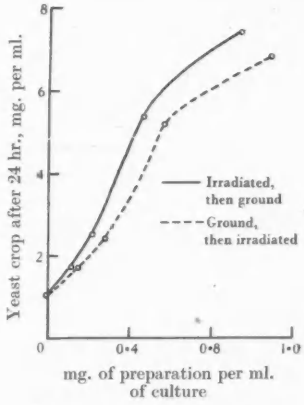

Fig. 3. Effect of killing cells by grinding before irradiation.

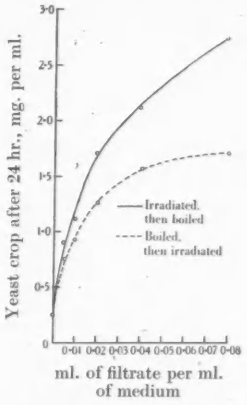

Fig. 4. Effect of killing cells by boiling before irradiation.

The products from cells irradiated while alive were slightly more active, both on a weight basis and equivalent filtrate volume basis, but the differences were small. Fig. 3 is a representative assay. The weight yields averaged from 20 to 30 mg./ml. in various experiments (compared with 2–5 mg./ml. in techniques in which unfragmented cells were used). Considerable difficulty was encountered in fragmenting all the cells by the method used.

The higher yields in the case of cells irradiated while alive seem to indicate that mechanism *c* was involved in the production of active factors.

6. *Comparison of yields from cells killed by boiling before irradiation and boiled after irradiation.* As an alternative to the above procedure, yields from cells killed by boiling were compared with yields from cells irradiated while alive. Yeast was suspended in isotonic NaCl, at 100 g./l. and divided into two aliquots. One of these was boiled for 3 min. to kill the cells. Both were then irradiated in quartz test tubes with full ultraviolet until all cells in the unboiled suspension were killed (methylene blue test). The irradiation time in the experiment illustrated in Fig. 4 was 4·25 hr. The distance from the quartz mercury arc was 15 cm. Following irradiation, the unboiled suspension was boiled for 3 min. Both suspensions were filtered through Berkefeld filters, and their filtrates were dried and assayed.

The suspensions irradiated before boiling gave more potent filtrates. This may be interpreted as indicating that proliferation-promoting factors are produced in living cells during injury (mechanism *c*) or that previous boiling interferes with extraction and results in lower yields from suspensions boiled before irradiation. The later interpretation does not seem to explain the phenomenon completely, however, because the filtrates from the suspensions irradiated while alive were more potent per unit weight as well as per unit volume.

Discussion

The question as to whether the increased proliferation-promoting effect of intercellular fluids from injured cells may be attributed in part to factors produced by living cells as a specific response to injury is an extremely difficult one to answer with finality. Certainly, there exist in yeast cells many substances stimulating yeast growth, such as amino-acids, pantothenic acid, vitamin B_1, vitamin B_6, biotin etc., as has already been pointed out repeatedly in connexion with this problem [Cook *et al.* 1940; Davidson, 1940; Loofbourow & Morgan, 1940]. When cells are injured or killed, the release into the intercellular fluids of such substances present in the uninjured cell might be expected to complicate any attempt to determine the presence of factors specifically produced as a result of injury. Nevertheless, the greater potencies of preparations from cells injured slowly rather than killed rapidly, the rapid increase in such factors in the intercellular fluids before appreciable killing, the appearance of such factors in suspensions of living cells subjected to CO_2, and finally, the greater yields from cells fragmented or killed by boiling after rather than before prolonged injury all suggest that living, injured cells do produce proliferation-promoting factors (intercellular wound hormones) while undergoing injury.

Various evidence that proliferation-promoting intercellular hormones from injured cells are related to nucleic acids has been presented [Loofbourow, Cook & Stimson, 1938; Cook *et al.* 1939]. The results of Davidson [1940] in which assays of extracts from irradiated cells showed markedly higher nucleoside- and free purine-nitrogen than extracts from non-irradiated or autoclaved cells suggest this same conclusion. It is difficult to explain Davidson's results as entirely due to increased membrane permeability following irradiation or to the release of dead cell disintegration products as a result of irradiation, for the nucleoside- and purine-nitrogen was three times as great in his filtrates from irradiated as in his filtrates from autoclaved cells, whereas the dry weights of the materials obtained were roughly 3 to 2 in favour of the autoclaved cell extracts. These results seem to us an argument for the production of nucleic-acid-like materials as a specific response to injury.

The suggestion is frequently made that our intercellular wound hormones may be synthesized by a specific photochemical process in the cell. The fact that

the active materials are obtained from cells injured in other ways and the indications that they are produced in living cells rather than by an effect of ultraviolet on the dead constituents of protoplasm are in our opinion arguments against this possibility.

Summary

In order to determine whether the increase in the proliferation-promoting effect of intercellular fluids following cell injury is due to (*a*) release of dead cell disintegration products, (*b*) increased permeability of the cell membrane or (*c*) formation of proliferation-promoting intercellular hormones by cells as a specific response to injury, the following types of experiments were tried.

(1) A comparison was made of the effects of rapid killing and of slow injury. The yield of proliferation-promoting intercellular hormones was, within limits, closely proportional to the time required to kill the cells.

(2) The yield of active factors was compared with the number of cells killed and found to increase rapidly before appreciable killing took place.

(3) The yields from cells injured in various media were compared and found to be highest when the medium contained carbohydrate, nitrogenous substances, phosphoric acid etc.

(4) CO_2 was found to increase the yield of active factors in the intercellular fluids without killing the cells.

(5) Cells exposed to lethal ultraviolet before grinding yielded more potent preparations than cells irradiated after grinding.

(6) Cells irradiated with lethal ultraviolet after they were killed by boiling yielded less potent preparations than those irradiated before boiling.

The results suggest that in addition to possible factors arising in the intercellular fluids as a result of mechanisms *a* and *b*, at least part of the potency must be attributable to factors arising from mechanism *c*.

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64. ENZYMIC DECOMPOSITION OF AMINO-SUGARS

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CONTINUING the study of the biochemical properties of cartilage and of other tissues which make up the joint [Lutwak-Mann, 1940, 1], experiments were carried out with chondroitin and mucoitin sulphuric acids, both of which occur in joint tissues (cartilage, tendons, mucous membranes). However, preliminary experiments have shown that comparatively little progress could be made in this field unless more information was available about the components of these complex substances and in particular, about the amino-sugars. In this paper results are given which describe the behaviour of amino-sugars under the influence of enzymes in animal tissues, yeast and bacteria. A preliminary account was given to the Biochemical Society [Lutwak-Mann, 1940, 2].

Materials

The following substances were used as substrates: glucosamine HCl, *N*-acetylglucosamine made according to White [1940], chondrosamine (galactosamine) HCl obtained by acid hydrolysis of chondroitin sulphuric acid, glucosaminic acid prepared from glucosamine by oxidation with HgO [Pringsheim & Ruschmann, 1915], chondroitin sulphuric acid from cartilage and from tendons made according to the method of either Levene & La Forge [1913] or Meyer & Smyth [1937].

For most experiments the animal tissues were used in the form of tissue slices but in some instances the tissues were minced and reduced to a pulp. There were also a few experiments where amino-sugars were fed or injected into animals (rats).

Yeast and bacteria were used as washed suspensions and only occasionally in the form of cell-free extracts.

Methods

Several methods were used to study the changes which amino-sugars undergo in the presence of various tissues and cells. Changes in the gas uptake or output were measured manometrically, using Barcroft differential manometers with side bulbs and gas outlets. In addition, the rate of decomposition of amino-sugars was estimated colorimetrically by the method of Elson & Morgan [1933]. The reducing sugar values were determined by the method of Hagedorn & Jensen [1923]. In all experiments the deamination of the amino-sugars was also studied and the NH_3-N was determined using the Parnas & Heller apparatus [1924]. The content of amino-N was occasionally estimated in the Van Slyke apparatus. It may be stated here that glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine were tested and found to yield no NH_3 in the presence of alkalis like borax, MgO, or $NaHCO_3$, which were used for the distillation of NH_3.

For the most part, rat and rabbit tissues were used, but some experiments were done with tissues of other animals (pig, horse, cat, guinea-pig).

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Experimental

A. *Behaviour of amino-sugars under the influence of animal tissues.* Increases in O_2 uptake and formation of NH_3 were invariably observed when amino-sugars were added to tissue slices from the following organs: kidney (cortex), testis, brain (cortex). Ovaries, retina and lung also showed certain rather small effects. On the other hand, negative results were obtained with liver, muscle (skeletal and heart), mucous membranes (intestine, joint), cartilage, spleen, red cells, plasma and synovial fluid. In Table 1 experiments are given which show the activities of a number of tissues towards glucosamine HCl, chondrosamine HCl and *N*-acetylglucosamine.

Table 1. *Oxidation and deamination of amino-sugars by animal tissues*

120 mg. wet wt. tissue slices. Ringer-phosphate *p*H 7·6; 10 mg. substrate. Barcroft manometers filled with O_2; KOH in inner tube.

			Glucosamine HCl		Chondrosamine HCl		*N*-acetyl-glucosamine	
Tissue	Exp.	Time hr.	μl. O_2*	μg. NH_3-N	μl. O_2	μg. NH_3-N	μl. O_2	μg. NH_3-N
Testis (rat)	1	½	72	22	60	10	80	8
	2	1	190	38	88	19	110	12
	3	1	150	32	100	22	170	20
		2	240	48	170	30	208	28
	4	4	—	128	—	42	—	48
Kidney cortex (rabbit)	1	½	40	16	30	3	36	Trace
	2	1	160	28	80	12	120	12
		3	240	68	160	28	220	28
		4	410	140	270	48	—	46
Brain (rat)	1	1	68	20	42	13	18	Trace
		2	108	30	96	26	42	12
Brain (rabbit)	2	4	—	78	—	—	—	—
Retina (pig)		2	190	28	78	16	190	4
Ovaries (cat)		2	—	32	—	—	—	16
Lung (rabbit)		2	—	29	—	12	—	12

* Values for O_2-uptake in this and all other tables refer to the 'extra'-O_2 uptake due to the substrate.

It can be seen from Table 1 that there are differences in the behaviour of the various tissues towards the three amino-sugars. In kidney and in testis there is comparatively little difference in the O_2 uptakes due to glucosamine HCl and *N*-acetylglucosamine. However, the deamination of the latter compound is slower than that of glucosamine. The enzyme system present in brain cortex shows little activity towards *N*-acetylglucosamine. It may be mentioned here that when experiments were carried out with mixtures of glucosamine and acetylglucosamine, the rates of deamination of both substances remained unaltered. The decomposition of chondrosamine HCl in all tissues examined is less rapid than that of glucosamine.

Before describing in detail the properties of the enzyme system involved in the breakdown of amino-sugars, it should be stated that in animal tissues their deamination was found to occur only aerobically. In the absence of O_2 the amino-sugars appeared to be stable in the presence of animal tissues; yeast and bacteria, on the other hand, were found to be active anaerobically as well (*v. inf.*). A distinct fall in *p*H due to acid production accompanied the O_2 uptake; no free CO_2 appeared to be formed in the course of the reaction.

Optimum p*H*. The breakdown of amino-sugars depends greatly on the *p*H which affects the oxidation and the deamination equally, so that it was impossible to separate the two processes by altering the acidity. The optimum *p*H was found to lie between 7·4 and 7·8. At *p*H 6·5 the reaction was very slow and below *p*H 6·0 it did not occur. Besides the phosphate buffer, *p*H 7·6, which was used throughout most of this work, borate, veronal and bicarbonate + CO_2 buffers were also tried. In all these buffers the reaction was slightly slower than in phosphate. In the presence of bicarbonate alone, both the O_2 uptake and the NH_3 production, though very rapid at the beginning, fell off nearly completely in the course of 30 min.

Comparison between the rate of O_2 uptake, NH_3 production and glucosamine disappearance. The experiments with animal tissues indicated that the O_2 uptake preceded the NH_3 formation, and similar results were obtained with yeast and bacteria, as will be seen later.

The results in Table 1 show that considerable amounts of O_2 were taken up in the course of amino-sugar breakdown, before any appreciable quantities of NH_3 could be detected. More detailed information can be gathered from the data in Tables 2–4. The experiments in Table 2 show that glucosamine added to

Table 2. *Rate of glucosamine disappearance and NH_3 formation*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	mg. glucosamine HCl found: By determination of reducing sugar	mg. glucosamine HCl found: By colorimetric method	μg. NH_2-N formed
Testis (rat)	4·8 mg. (≡312 μg. NH_2-N)	2½	2·5	3·0	62
		3½	2·0	1·7	85
Testis (rat)	3·0 mg. (≡195 μg. NH_2-N)	2	1·7	—	70
		3	1·38	—	85
Kidney (rabbit)	4·14 mg. (≡269 μg. NH_2-N)	1½	—	2·5	55
		3	—	1·8	100
Kidney (rabbit)	3·8 mg. (≡247 μg. NH_2-N)	¾	2·4	—	16
		1½	1·4	—	56
		3½	1·1	—	88
Kidney (rabbit)	4·0 mg. (≡260 μg. NH_2-N)	½	—	3·1	24
		2	—	1·9	70
		3½	—	1·42	104

tissue slices under aerobic conditions disappears fairly rapidly during the first 60–90 min., as shown both by the colorimetric and reduction values. The NH_3 liberation is very slow during this initial phase. In the later stages of the reaction the rate of amino-sugar disappearance falls considerably while the NH_3 production increases. However, even after prolonged periods of incubation, when more than ¾ of the substrate added has disappeared, the NH_3 values correspond only to about ½–⅔ of the amino-sugar used up during this period. In Table 3, in addition to the amounts of glucosamine used up and the NH_3 liberated, data are also given for the amount of O_2 taken up, while Table 4 illustrates the effect of *p*H on the course of glucosamine decomposition.

These experiments suggested the possibility of an oxidation product being first formed from the amino-sugar and then undergoing deamination. The decrease in *p*H which, as was mentioned previously, accompanied the O_2 uptake made it probable that this intermediary oxidation product would be an acid. It was thought that glucosaminic acid might be the substance formed under

Table 3. *O_2 uptake, glucosamine disappearance and NH_3 production*

Tissue	Glucosamine HCl added	Aerobic incubation time hr.	O_2-uptake due to glucosamine μl. O_2	mg. glucosamine HCl found	μg. NH_3-N formed
Testis (rat)	2 mg. ($\equiv$130 μg. NH_2-N)	1½	140	0·78	40
Brain (rabbit)	2 mg. ($\equiv$130 μg. NH_2-N)	1½	180	0·67	28

Table 4. *Effect of* p*H on decomposition of glucosamine*

Testis (rat). Added 5 mg. glucosamine HCl ($\equiv$358 μg. NH_2-N). Aerobic incubation.

Time (hr.)	*p*H	mg. glucosamine-HCl found: By determination of reducing sugar	mg. glucosamine-HCl found: By colorimetric glucosamine estimation	μg. NH_3-N formed
1½	7·6	3·7	3·3	55
	6·6	4·1	3·7	29
3½	7·6	2·5	2·2	130
	6·6	3·5	3·3	46

these circumstances. To test this possibility glucosaminic acid was prepared from glucosamine, but when tested with animal tissues it was found to be completely stable, both with regard to NH_3 formation and to O_2-uptake. The addition of glucosaminic acid to any of the amino-sugars tested in no way interfered with their usual course of breakdown. These results seemed to disprove the assumption that glucosaminic acid was the product responsible for the NH_3 formation from amino-sugars; other experiments which will be referred to below make its formation unlikely in the course of amino-sugar decomposition by the enzyme system described in this paper.

Effect of various substances on the oxidation and deamination of amino-sugars. It was stated before that, except in brain cortex, there is little difference in the rate of O_2-uptake due to glucosamine and *N*-acetylglucosamine, but that the deamination of the acetylated compound is generally very slow. It was thought therefore that this difference in the rates of deamination of the free and the acetylated glucosamine, could be utilized to prove the enzymic conversion of glucosamine into an acetyl (or other) derivative.

With this possibility in mind, the rate of NH_3 formation from glucosamine was examined in the presence of several substances which were considered likely to yield derivatives able to block the NH_2 group. Table 5 shows the effect of several organic acids, aldehydes, alcohols and sugars on the deamination. It was found that some of the substances used decreased not only the deamination of the amino-sugar but also the 'blank' NH_3 formation, which means that they have an inhibitory effect upon deaminases generally.

Among the acids tested, oxaloacetic acid showed a fairly strong inhibition of the NH_3 production from glucosamine, especially if used in high concentration. On the other hand, acetic, lactic and pyruvic acids caused not more than *c.* 10 % decrease. Butyric and β-hydroxybutyric acids showed a somewhat more marked effect, while oxalic acid had no influence.

The addition of acetaldehyde and methylglyoxal depressed the deamination to a moderate degree, but with *dl*-glyceraldehyde a much more pronounced effect was obtained, particularly if kidney cortex slices were used as the source of enzyme (Table 6).

Table 5. *Effects of various substances on the deamination of glucosamine*

			μg. NH_3-N formed				
			No additions		With additions		
Tissue	Exp.	Time hr.	Tissue alone	Tissue + glucosamine	Tissue alone		Tissue + glucosamine
Kidney	1	2	28	80	22	Acetate *M*/10	70
	2	2½	28	100	28	Butyrate *M*/20	60
		2½	28	100	28	β-Hydroxybutyrate *M*/20	85
	3	3	36	130	35	Oxaloacetate *M*/40	62
		3	36	130	28	Oxaloacetate *M*/15	32
		3	36	130	32	Oxalate *M*/20	110
Testis	1	1	15	65	13	Pyruvate *M*/20	60
		1	15	65	12	Lactate *M*/20	52
Brain	1	2	30	56	40	Formate *M*/20	60
		2	30	56	30	Citrate *M*/20	48
Kidney	1	2	26	90	20	Acetaldehyde *M*/20	78
		2	26	90	18	Methylglyoxal *M*/30	50
		2	26	90	15	Glyceraldehyde *M*/20	30
Kidney	1	3	38	122	20	Glycerol *M*/40	77
	2	2	22	90	22	Menthol *M*/50	78
		2	22	90	26	Borneol *M*/50	90
Testis	1	3	30	112	20	Glucose *M*/20	42
	2	3	37	96	8	Glucose *M*/5	20
Kidney	1	1½	22	60	18	Glucose *M*/5	30
	2	3	32	125	20	Glucose *M*/20	56
		3	32	125	26	Cori ester *M*/10	70
		3	32	125	27	Fructose *M*/10	73
		3	32	125	36	Galactose *M*/10	120
		3	32	125	36	Sucrose *M*/8	140
Brain	1	2	15	49	4	Glucose *M*/8	22
	2	2½	40	80	41	Gluconate *M*/8	76
		2½	40	80	44	Glucurone *M*/10	78

Table 6. *Effect of glyceraldehyde on glucosamine deamination in various tissues*

			μg. NH_3-N formed				
			No additions		With glyceraldehyde		
Tissue	Exp.	Time hr.	Tissue alone	Tissue + glucosamine	Tissue alone		Tissue + glucosamine
Kidney	1	2	22	84	13	Glyceraldehyde *M*/50	37
	2	1½	20	66	18	Glyceraldehyde *M*/100	30
			20	66	16	Glyceraldehyde *M*/30	22
Testis	1	2½	36	85	27	Glyceraldehyde *M*/50	48
	2	1½	15	47	10	Glyceraldehyde *M*/60	31
Brain	1	1½	26	40	23	Glyceraldehyde *M*/50	32

When various sugars were examined for their ability to influence the deamination of glucosamine, it was found that glucose alone among several sugars tested strongly interfered with the amino-sugar deaminase. It was possible, by using higher concentrations of glucose, to prevent the deamination of glucosamine nearly completely (Tables 5 and 7). Except for glucose-1-phosphoric acid (Cori ester), none of the other sugars could be compared with glucose in its effect upon the deaminating enzyme.

It seemed desirable to investigate more closely the behaviour of amino-sugars in the presence of glyceraldehyde and glucose, not only in connexion with their

effects upon the deamination but also with regard to the O_2-uptake. The colorimetric estimation of glucosamine disappearance was, however, impossible in the presence of glyceraldehyde, owing to the fact that the presence of this aldehyde prevented the formation of colour in the method of Elson & Morgan. Similarly, it was impracticable to attempt to assess the reducing sugar values for the amino-sugar in the presence of glucose; in the colorimetric method glucose caused no interference.

It can be seen from Table 7 that glucose had a more pronounced effect on glucosamine deamination than glyceraldehyde but that glyceraldehyde inhibited the O_2-uptake more than glucose. The same table contains also data obtained with lactate and pyruvate, which had little effect upon either the oxidation or the deamination of glucosamine.

Table 7. *Effects of glucose, glyceraldehyde, lactate and pyruvate on the O_2 uptake and NH_3 formation*

Tissue	Time		μl. O_2 taken up	μg. NH_3-N formed
Testis	2 hr. 30 min.	No addition	290	78
		Glucose $M/15$	205	29
Kidney	1 hr. 30 min.	No addition	172	48
		Glucose $M/12$	119	12
Testis	1 hr. 30 min.	No addition	210	42
		Glyceraldehyde $M/60$	38	21
Testis	50 min.	No addition	120	30
		Lactate $M/20$	90	28
		Pyruvate $M/20$	148	33

In some of these experiments the content of amino-N was estimated in the samples at the beginning and end of the experiment, both without and with the addition of substances like glyceraldehyde, glucose, oxaloacetate, lactate and pyruvate. But the results obtained completely failed to produce any evidence that the glucosamine amino-group was blocked, as might have been expected if acetylation or a similar process had taken place during the incubation of glucosamine with these substances.

To ascertain whether the effect of glucose on the glucosamine deamination was due to glucose itself or to its breakdown products, experiments were carried out in which the tissue slices were incubated for a time with glucose, either in air or anaerobically, before the amino-sugar was added. From the data in Table 8 it appears that the presence of glucose oxidation products had practically the same effect on the deamination of glucosamine as glucose alone; there was, however, a small increase in the amount of O_2 used up. When the tissue was kept with glucose in N_2, before the addition of glucosamine, the results did not differ

Table 8. *Effect of glucose and of its products on glucosamine oxidation and deamination*

Tissue slices (testis) incubated for 1 hr. with glucose in O_2 (*c*) or in N_2 (*d*), before addition of glucosamine.

		μl. O_2/1 hr.		μg. NH_3-N/1 hr.	
		Exp. 1	Exp. 2	Exp. 1	Exp. 2
(*a*) Glucosamine		156	160	52	61
(*b*)	+glucose (no incub.)	100	118	20	13
(*c*)	+glucose (O_2 incub.)	180	195	18	10
(*d*)	+glucose (N_2 incub.)	94	100	20	4

from those obtained when glucose and glucosamine were added simultaneously to the tissue slices.

Interesting results were obtained when glucose and galactose were compared in their effects on the oxidation and deamination of chondrosamine. The experiment in Table 9 shows that while galactose had comparatively little effect on the deamination of this amino-sugar, it interfered more with its oxidation than glucose.

Table 9. *Effects of glucose and galactose on chondrosamine breakdown*

Tissue		μl. O_2 taken up in 90 min.	μg. NH_3-N formed in 90 min.
Kidney	Chondrosamine	280	52
	+glucose $M/10$	210	20
	+galactose $M/10$	170	40

Poisons and inhibitors. With the intention of obtaining some indication as to the type of reaction involved in the enzymic decomposition of amino-sugars, several substances were tested with regard to their effects on the enzyme system and the results are collected in Table 10. The experiments in this table were carried out using kidney cortex slices, but analogous results were obtained with other active tissues.

Table 10. *Effects of poisons and inhibitors on glucosamine oxidation and deamination*

	μl. O_2 taken up/100 min.	μg. NH_3-N formed/100 min.
Without additions	230	70
With		
Iodoacetate $M/100$	0	0
Fluoride $M/50$	156	44
Arsenate $M/75$	130	33
Cyanide $M/250$	56	52
Cyanide $M/75$	32	29
Azide $M/75$	190	58
Malonate $M/100$	200	67
Cinchophen $M/10$	0	0
Salicylate $M/50$	180	43
Toluene	0	0
Sulphapyridine	248	75
Merthiolate 1 : 20000	0	0

It was interesting to discover that the reaction under investigation is extremely sensitive to iodoacetic acid (IA). Both oxidation and deamination of amino-sugars were abolished by this poison. Smaller effects were obtained with NaF and arsenate, while malonate and azide caused no inhibition at all. KCN largely prevented the oxidation, but had comparatively little effect on the NH_3 formation. Salicylic acid was found to be a mild inhibitor, but cinchophen (phenyl cinchoninic acid) suppressed completely the activity of the amino-sugar enzyme system. Organic solvents like alcohol, acetone, ether and toluene destroyed the enzyme, even in low concentrations. It was also inactivated in the presence of merthiolate. On the other hand, sulphanilamide and sulphapyridine had no influence on the reaction. On several occasions the tissues used for the experiments with amino-sugars, were removed from animals which had received sub-lethal doses of either sulphapyridine or sulphanilamide; yet the enzyme system responsible for the decomposition of amino-sugars remained entirely unaffected.

The effect of semicarbazide and dimedone upon the rate of glucosamine breakdown was also tested; no significant changes, however, were observed, as might have been the case if ketones or aldehydes had been produced in the course of the reaction. The lack of effect with these 'trapping' substances agreed well with the negative results obtained for bisulphite-binding substances (*vide infra*).

Tissues other than kidney, testis and brain. It was mentioned previously that a slight O_2 uptake and deamination of amino-sugars could be observed with ovaries, retina and lung tissue. Among other animal tissues and fluids which were tested in various ways and found to contain no enzymes capable of either the oxidation or deamination of amino-sugars, were liver, muscle, intestine, cartilage, blood (whole and haemolysed), serum and synovial fluid.

Particular attention was paid to liver as this tissue is well known to possess enzymes which might be related to the system dealing with amino-sugars, such as glucose oxidase and amino-acid oxidase. However, in all experiments with liver tissue, used either in the form of slices or pulp, neither oxidation nor deamination of amino-sugars took place. The absence of deaminase activity was not due to substitution of the amino-group, because it was found after prolonged incubation of glucosamine with liver that the amino-group remained free even when such substances as acetate, lactate, pyruvate etc., were added.

It may be of some interest to mention here that the addition of amino-sugars greatly interfered with the oxidation of glucose by liver slices (Table 11). A similar effect of amino-sugars upon glucose oxidation was also observed in testis slices; in this instance there was, of course, a mutual interference of glucose and amino-sugar as described on p. 615.

Table 11. *Effect of amino-sugars on glucose oxidation in liver*

	μl. O_2 taken up/70 min.
Glucose	205
+ glucosamine	100
+ chondrosamine	130
+ *N*-acetylglucosamine	84
Glucosamine	0
Chondrosamine	0

It appeared fairly probable from the results obtained with liver that this organ is devoid of enzymes acting on amino-sugars. It seemed, however, worth while to investigate whether the apparent stability of the amino-sugars in liver was not due to the presence of substances capable of suppressing their decomposition.

Liver slices were incubated aerobically for 1–2 hr. in Ringer-phosphate; the tissue was then removed and the liquid was heated for 5 min. to 80°. When this heat-inactivated fluid was added to kidney cortex slices, it considerably retarded the deamination of glucosamine. Similar effects were obtained by adding the extract from liver to brain or testis slices. If, however, the liver slices were incubated anaerobically instead of aerobically, the effect of the heat-inactivated extract was much less conspicuous. Further experiments of this kind showed that the liver factor responsible for the suppression of amino-sugar deamination, is dialysable and heat-stable and also that it is not formed aerobically at *p*H below 6·0 (Table 12). On the other hand, its formation in the liver was not prevented by KCN, phloridzin or semicarbazide. Heat-inactivated extracts from kidney or testis slices, or boiled yeast juice, failed to produce any marked effect on the NH_3 production from amino-sugars.

Table 12. *Effect of heat-inactivated liver extract on the deamination of glucosamine by kidney cortex*

	μg. NH_3-N produced/1 hr. 30 min.
Glucosamine	68
+ liver extract, aerob., *p*H = 7·4	22
+ liver extract, dialysed	50
+ liver extract, aerob., *p*H = 4·8	60
+ liver extract, anaerob., *p*H = 7·4	42

In experiments with muscle or mucous membranes, it was necessary to mince the tissues and to use them in the form of a pulp. As stated previously, negative results were obtained with these tissues, except for some very slight anaerobic oxidation of glucosamine by washed heart muscle mince in presence of cozymase and methylene blue (Table 15). In this connexion it should be stated that when active kidney cortex or testis slices were broken up by grinding in Ringer-phosphate and made into a thick pulp, their activity towards amino-sugars was considerably reduced (Table 13). It would seem therefore that the enzyme system dealing with amino-sugars depends largely upon the tissue structure being preserved intact. Completely negative results were obtained with a kidney cortex acetone powder preparation, although this preparation was very active towards *d*-alanine. Also a preparation of testicular diffusing factor (obtained through the kindness of Dr Bacharach) appeared devoid of any power to oxidize or to deaminate amino-sugars.

Table 13. *Decomposition of amino-sugars by tissue slices and by pulp*

Tissue	Preparation	Substrate	μl. O_2/2 hr.	μg. NH_3-N/2 hr.
Kidney cortex	Slices	Glucosamine	260	79
		N-acetylglucosamine	190	21
	Pulp	Glucosamine	56	38
		N-acetylglucosamine	Not measured	Trace
Testis	Slices	Glucosamine	190	88
		N-acetylglucosamine	142	34
	Pulp	Glucosamine	Very small	52
		N-acetylglucosamine	Very small	18

Attempts to identify the reaction products. The distinct fall in *p*H accompanying the breakdown of amino-sugars, made probable the formation of an acid in the course of the reaction. Estimations of lactic and acetic acids were negative. The presence of succinic acid was made improbable by experimental results described below.

Estimations of bisulphite-binding substances [Clift & Cook, 1932] gave negative results. No substances were formed which would yield derivatives with 2:4-dinitrophenylhydrazine.

A special series of experiments was carried out to investigate more thoroughly the formation of glucosaminic acid. It was mentioned already that glucosaminic acid was found to be remarkably stable in the presence of animal tissues and therefore it could hardly be regarded as the (hypothetical) oxidation product from glucosamine which undergoes deamination. Nevertheless, it seemed important to examine whether glucosaminic (or chondrosaminic) acid was formed from the amino-sugars under the experimental conditions described in this paper.

This was done in the following manner. It was found that glucosaminic acid yields CO_2 readily in the presence of chloramine-T at *p*H 4·6, in quantities roughly proportional to the amount of the acid, while glucosamine causes only a very slow gas production under similar conditions. The CO_2 evolution can easily be measured manometrically (Table 14).

Table 14. *Behaviour of amino-sugars and glucosaminic acid in the presence of chloramine-T*

	μl. CO_2/20 min., 38°, *p*H = 4·6
Glucosamine HCl, 2 mg.	15
Chondrosamine HCl, 2 mg.	12
Glucosaminic acid, 2 mg.	120
Glucosaminic acid, 4 mg.	195

The technique adopted for the estimation of glucosaminic acid largely followed the method described by Cohen [1940]. It was found necessary to remove NH_3 formed from glucosamine in the course of the enzymic reaction, in order to avoid N_2 formation by the chloramine-T treatment. This was conveniently accomplished by distillation in the Parnas-Heller apparatus.

Tissue slices (250 mg. wet wt., total volume of solution 3 ml.) were incubated with a known quantity of glucosamine in the usual manner. O_2 uptake and NH_3 formation were estimated and at various intervals the reaction was stopped by heating the sample to 90° for 3 min. After the removal of NH_3 and the precipitation of proteins by tungstic acid, the filtrate was condensed at 100° to half its original volume. The amount of fluid taken for the final chloramine-T experiment was chosen so as to contain between 2 and 4 mg. of glucosaminic acid. Numerous controls were carried out to ascertain that glucosaminic acid added to the tissue under the conditions referred to could be recovered without loss in spite of the prolonged preparatory manipulations.

However, from a series of experiments carried out with glucosamine and chondrosamine HCl, both alone and in the presence of glucose, it appeared certain that glucosaminic acid was not formed under these conditions, at least in any appreciable quantities. Similar negative results were also obtained using *B. coli* instead of the animal tissues.

The following experiments were carried out in yet another attempt to identify the reaction products formed during the breakdown of amino-sugars. Minced and thoroughly washed muscle pulp was prepared, such as is known to contain several dehydrogenases. Using this enzyme preparation, the effect was studied on the methylene blue reduction time of the addition of a heat-inactivated extract from tissue slices which had been allowed to act for a considerable time on amino-sugars. Table 15 gives the results of some of these Thunberg experiments. It can be seen that (*a*) glucosamine itself is very slowly oxidized by heart muscle enzymes in the presence of cozymase, and (*b*) in the tissue slices extract containing the reaction products from amino-sugars, there is a substance which in the presence of cozymase definitely accelerates the reduction of methylene blue. There are two substrates which do not require cozymase for their anaerobic oxidation with methylene blue by muscle enzymes: succinic and α-glycerophosphoric acids. These two can therefore be excluded as possible intermediary or end-products of amino-sugar breakdown. The formation of α-glycerophosphate was already unlikely, because separate experiments showed no evidence of phosphorylation being involved in the amino-sugar decomposition.

Table 15. *Effect of muscle dehydrogenases on the reaction products of amino-sugar breakdown*

0·3 g. muscle dehydrogenase preparation, 0·6 mg. cozymase, 1 ml. *M*/2000 M.B., *p*H = 7·4.

	Reduction time, min.
Heart muscle prep.	∞
+ cozymase	95
+ glucosamine	∞
+ cozymase + glucosamine	28
+ cozymase + chondrosamine	70
+ extract from tissue slices	80
+ cozymase + extract from slices	45
+ extract from slices incubated with glucosamine	55
+ cozymase + extract from slices incubated with glucosamine	12
Skeletal muscle prep. + cozymase	50
+ cozymase + *N*-acetylglucosamine	40
+ cozymase + extract from slices	38
+ cozymase + extract from slices incubated with *N*-acetylglucosamine	14

Experiments with chondroitin sulphuric acid. Comparatively few experiments were carried out using this compound as substrate for tissue or bacterial enzymes; in fact, their variability was the main cause why they were abandoned for the time being and the research on amino-sugars was taken up instead.

Chondroitin Na-sulphate was added to tissue slices (kidney, testis) and incubated for 2, 4 and 6 hr. at 37°, at *p*H values varying between 6·0 and 8·0. Afterwards the supernatant fluid was examined for the presence of amino-sugars. Traces of amino-sugar could usually be detected but the amount varied considerably and it appeared probable that this was due to a further decomposition of the amino-sugar set free from chondroitin sulphuric acid by the tissue enzymes. This assumption was confirmed by the results of the work reported in this paper.

Attempts were also made to assess the rate of amino-sugar liberation during acid hydrolysis of chondroitin sulphuric acid. Use was made of the enzymes present in tissue slices to indicate the presence of amino-sugar. For this purpose, samples of chondroitin sulphuric acid were hydrolysed in sealed tubes at 100° in *N* HCl for 1, 2, 4 and 12 hr. They were carefully neutralized and freed from NH_3. They were then added to testis or kidney cortex slices and incubated aerobically in the usual manner. Production of NH_3 indicated the presence of amino-sugar, as chondroitin sulphuric acid itself gave rise to no NH_3 formation. In this way it was found that after 60 to 90 min. of acid hydrolysis chondroitin sulphuric acid yields a considerable amount of amino-sugar in a form in which it can be attacked by the tissue enzymes. An inconvenient complication of these experiments was caused by the rather high NaCl concentration of the hydrolysed and neutralized samples. It was ascertained in separate experiments that *N* NaCl or KCl completely inactivates the system acting on amino-sugars. It was therefore necessary to dilute the hydrolysed samples very considerably to diminish the salt concentration and thus only comparatively small fractions of the amino-sugar present were used for the final enzymic experiment.

Experiments with animals. One of the main obstacles, so far, to the study of amino-sugar metabolism is due to the difficulty of quantitative estimation of amino-sugar in urine. It appears probable that urea is the substance which interferes with the colorimetric estimation of amino-sugar in urine, as it was

found that small amounts of urea give the same orange (instead of pink) colour with the Ehrlich reagent as is obtained with dilute urine.

As no successful and simple way could be found to apply the Elson & Morgan method to estimations in urine, it was decided to study instead the effect of amino-sugars on the NH_3-N content of the urine in animals which were known to possess organs able to deaminate amino-sugars. Glucosamine HCl (1 mg./g. body weight) in Ringer was injected subcutaneously into rats (180 g.) while Ringer alone was injected into control animals. The following method of urine collection was adopted. The food was removed early on the day of the experiment and urine was collected for some 10 hr. Then followed the injection and a further collection was made for 10–12 hr. Food was then restored and urine was collected for a further 4–6 hr. In all samples the total volume, *p*H and the NH_3-N were then estimated. Table 16 gives the results of some of these experiments. It can be seen that there were considerable variations both in the volume of urine excreted in a given time and in the NH_3-N content of the urine samples, even before the injection. In the samples following the injection there was a slight increase in the NH_3-N content of the urine in the animals which received glucosamine HCl, but this was very small indeed if compared with the NH_3-N content of the amino-sugar injected. There were no significant changes in the *p*H of the urine samples after the injection of the amino-sugar. It would seem that, at least in rats, the amino-sugars were prevented from complete deamination, although *in vitro*, in the presence of rat tissues like kidney or testis, they would have lost their amino-group in the course of prolonged incubation.

Table 16. *Effect of subcutaneous injection of glucosamine on the NH_3 content of urine in rat*

	Volume (ml.)			*p*H			mg. NH_3-N in total urine		
Urine sample	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Fasting animals:									
7 hr. before injection of saline	3	15	1	6	6·9	5·9	6·00	4·95	1·40
7 hr. before injection of glucosamine	7	18	1·3	5·9	6·6	6·0	6·16	9·00	0·80
Fasting animals:									
12 hr. after injection of saline	2·6	8	3	6	6·6	5·8	2·47	2·80	4·20
12 hr. after injection of glucosamine	3·4	6	9	5·9	6·5	6·2	3·40	6·00	7·20
Fed animals, 10 hr. after above collection:									
Saline-injected animals	11	8·5	—	7·3	7·5	—	2·53	3·65	—
Glucosamine-injected animals	16·5	6·5	—	7·0	6·9	—	2·55	3·30	—

It seems worth while to report briefly certain other experiments, which although interesting cannot at present be extended owing to the difficulties in obtaining large quantities of dried egg white. Male albino rats were kept on the 'egg white injury'-producing diet [György, 1939]. After a period of about 120 days they developed the typical skin and spectacled eye condition characteristic of egg white injury. Some of these animals were given 20 mg. of glucosamine HCl per day for a week, during which period they began to show a definite improvement such as growth of hair on the most affected skin areas as well as a distinct clearing up of the eye exudates. Their weight was maintained at a constant level. Chondrosamine HCl tested under similar conditions failed to produce any effect at all. However, after some 8 days of improvement due to glucosamine its effect faded and a relapse followed, which was not relieved by further doses of glucosamine HCl.

In a different series of experiments the effect of glucosamine feeding was investigated on B_2-deficient rats. Here however, no visible results of any kind were obtained. The tissues of both egg white-fed and B_2-deficient rats possessed the enzyme system concerned with the breakdown of amino-sugars.

B. *Yeast enzymes.* Several experiments were carried out using suspensions of baker's or brewer's yeast as the source of enzymes acting on amino-sugars. An enzyme system was found to exist in yeast which very slowly deaminated glucosamine and chondrosamine HCl in aerobic as well as in anaerobic conditions. The enzymes showed very little activity towards *N*-acetylglucosamine (Table 17). No free CO_2 appeared to be formed either in air or in N_2. As in animal tissues, the rate of oxidation exceeded the NH_3 formation; there was also the formation of acid reaction products which have not yet been identified.

Table 17. *Decomposition of amino-sugars by baker's yeast*

12 mg. yeast (dry wt.), 10 mg. substrate, pH = 7·6.

	Oxygen uptake μl. O_2/1 hr.	Aerobic NH_3 formation μg. NH_3-N/2½ hr.	Anaerobic NH_3 formation μg. NH_3-N/2½ hr.
Glucosamine-HCl	120	60	28
Chondrosamine-HCl	72	34	11
N-acetylglucosamine	50	28	8
Glucose	1340	—	—

The yeast enzymes showed the same behaviour towards poisons, e.g. iodoacetate and KCN, as described previously in connexion with animal tissues. The specificity of the glucosamine deaminase in yeast was proved by the fact that while it was completely inhibited in the presence of iodoacetate, this poison did not affect the deamination by yeast of other substrates such as adenosine or adenylic acid.

Cell-free extracts from fresh baker's yeast, as well as Lebedew maceration juice from dried brewer's yeast, were found to be very weak in activity towards amino-sugars.

C. *Bacterial enzymes.* For most experiments *B. coli* was used in washed suspensions, but a few experiments were also done using suspensions of *Str. faecalis* and *Proteus vulgaris*.

B. coli was found to contain a powerful enzyme system which at pH 6·8–7·8 rapidly oxidized and deaminated glucosamine HCl and, a little more slowly, chondrosamine HCl and *N*-acetylglucosamine (Table 18).

Table 18. *Decomposition of amino-sugars by bacteria*

2 mg. suspension (dry wt.), 10 mg. substrate, pH = 7·6.

	Glucosamine HCl	Chondrosamine HCl	*N*-acetyl-glucosamine
B. coli:			
μl. O_2/40 min.	180	110	130
μg. NH_3-N/40 min.: aerob.	98	62	80
μg. NH_3-N/40 min.: anaerob.	40	28	38
Str. faecalis:			
μl. O_2/50 min.	90	45	—
μg. NH_3-N/50 min.: aerob.	160	130	—
μg. NH_3-N/50 min.: anaerob.	140	41	—
Proteus vulgaris:			
μl. O_2/60 min.	68	90	—
μg. NH_3-N/60 min.: aerob.	18	29	—

The oxidation of amino-sugars was accompanied by acid formation. Also in anaerobic conditions *B. coli* deaminated the amino-sugars and produced acid reaction products from them. There was no CO_2 evolution in the course of either of these reactions.

Except for the ability to decompose the amino-sugars anaerobically, the bacterial enzyme system behaved in all respects like the animal system, i.e. the same substances were found to act as inhibitors and poisons in bacteria as described above for kidney slices or testis (Table 19).

Table 19. *Effects of glyceraldehyde, glucose, cyanide and iodoacetate on the decomposition of glucosamine by* B. coli

	μl. O_2/1 hr.	μg. NH_3-N/1 hr.: aerob.
Glucosamine	290	160
+ glyceraldehyde $M/60$	85	100
+ glucose $M/20$	188	36
+ cyanide $M/100$	60	130
+ iodoacetate $M/150$	0	0

The sequence of the processes in *B. coli* which cause the breakdown of amino-sugars appeared to be similar to that demonstrated for the animal enzymes, that is to say oxidation preceded deamination. By adding glucose to the bacterial suspension it was possible to diminish considerably the NH_3 liberation without much affecting the O_2 uptake. On the other hand, the addition of glucose to the culture medium of *B. coli* did not influence the amino-sugar deaminase in any marked way.

B. coli was also found to be capable of rapidly oxidizing the amino-sugars under anaerobic conditions in the presence of methylene blue as hydrogen acceptor. Table 20 A illustrates the results of experiments carried out with the Thunberg technique. It shows the effects of various poisons on the anaerobic oxidation, which are analogous with those obtained in aerobic conditions. Treatment of the bacterial suspension with toluene completely destroyed the enzyme activity.

Further experiments of this kind confirmed the assertion previously made that considerable amounts of the amino-sugar are oxidized before any large-scale deamination sets in. In the B section of Table 20 the results show that in the course of 16 min. roughly 3 mg. of methylene blue were reduced (or *c.* 2 mg. glucosamine HCl oxidized), but that the amount of NH_3 produced during this

Table 20. *Anaerobic oxidation of glucosamine by* B. coli

1 mg. bacterial suspension (dry wt.); 1 ml. $M/2000$ M.B.; pH = 7·4; total volume, 3 ml.

A.	Reduction time, min.
Blank	60
Glucosamine	4
Glucosamine, pH = 6·0	28
Glucosamine + iodoacetate $M/100$	49
+ fluoride $M/70$	37
+ cyanide $M/100$	5
+ toluene	65

B. 4 mg. bacterial suspension (dry wt.) in 3 ml. total volume.

	Reduction time, min.	μg. NH_3-N found
Blank + 2 mg. M.B.	70	24
Glucosamine + 1 mg. M.B.	4	40
+ 2 mg. M.B.	9	49
+ 3 mg. M.B.	16	80

period (0·056 mg. NH_3-N) corresponded to not more than half the amount of the substrate oxidized.

Various attempts were made to isolate the reaction products of glucosamine breakdown in *B. coli*, on lines similar to those adopted for animal tissues. It was established that there was no lactic or glucosaminic acid formation. The latter was found to be completely stable in the presence of bacteria.

Suspensions of *Str. faecalis* were found to be active towards the amino-sugars, but their oxidizing enzymes were less strongly developed than those of *B. coli*, while their deaminase was rather powerful, both in O_2 and in N_2.

Suspensions of *Proteus vulgaris* acted very slowly on glucosamine HCl both as regards O_2 uptake and deamination; they appeared, however, slightly more active towards chondrosamine HCl (Table 18).

Discussion

From the experimental data presented above some information can be gathered about the enzyme system capable of amino-sugar decomposition. Its fairly wide distribution, as shown by the fact that it occurs in animal tissues, yeasts and bacteria, is of particular interest when one bears in mind that amino-sugars form an essential part of various complex polysaccharides which seem to play an important part in many physiological processes.

There is evidence from the experiments described that the enzyme system concerned in the decomposition of amino-sugars is strictly specific for these substances. Its non-identity with the glucose oxidase was easily proved by the results obtained with liver. This organ was incapable of attacking the amino-sugars although it rapidly oxidizes glucose. Moreover, amino-sugars were found to interfere with the glucose oxidase; this phenomenon may perhaps be regarded as an instance of competitive inhibition.

Furthermore, there appears to be no relationship between the amino-sugar enzyme and the amino-acid oxidase; this can be simply shown using acetone kidney cortex preparations, which, though strongly active towards *d*-alanine, fail completely to attack the amino-sugars. It was also found that while iodoacetic acid completely stopped the breakdown of glucosamine in kidney cortex slices, it had comparatively little effect on the decomposition of *l*-alanine. The amino-sugar enzyme is also distinct from the tyramine oxidase, as shown by experiments with intestinal mucosa which oxidizes and deaminates the amine but does not act on amino-sugars.

By applying iodoacetic acid to yeast it was possible to show that the glucosamine deaminase is distinct from the enzyme which deaminates adenine derivatives.

The enzyme system concerned with the amino-sugars seems to be composed of two closely linked enzymes of which one is responsible for the oxidation and the other for the deamination of the substrate. The evidence for this view is based on the observation of the behaviour of the enzyme system in the presence of various substances. For instance, by the addition of glyceraldehyde or KCN, one can largely depress the oxidation without much affecting the deamination; on the other hand, glucose or oxaloacetate considerably diminish the amount of NH_3 produced, but have less effect on the O_2 uptake. The fact that the enzyme largely depends on the integrity of cell structure, makes it difficult to resolve this system into its components. As the experiments with cell-free extracts from tissues or from other sources were few, it was not possible to determine whether this enzyme required any coenzyme or what the nature of this coenzyme might be.

Generally speaking, of the three amino-sugars tested, glucosamine is oxidized and deaminated more rapidly than either chondrosamine or acetylglucosamine; this difference in the rates of breakdown exhibited by the enzyme preparations tested may be due to a greater affinity for glucosamine than for galactosamine. The presence of the acetyl group in *N*-acetylglucosamine proves an obstacle to the deaminase but interferes comparatively little with the oxidation. In spite of many attempts to bring about an enzymic acetylation of glucosamine, no positive results have been achieved so far. But the difference in the rates of deamination of glucosamine and acetylglucosamine may be very useful as an indication of the blocking of the NH_2 group.

The nature of the reaction products in addition to NH_3 formed from amino-sugars has yet to be discovered. So far, it seems certain that there is no lactic, acetic or succinic acid among the breakdown products; substances of aldehyde or ketone character are also absent. With regard to the mechanism of the enzymic decomposition it appears probable that the amino-sugars undergo first an oxidation and that it is this oxidation product which subsequently gives off NH_3. The experimental results indicate that glucosaminic acid is not formed in the course of glucosamine oxidation and it can therefore be excluded as the reaction product which undergoes deamination. It was interesting to note the considerable stability of glucosaminic acid in the presence of enzymes from various sources.

From the experiments with the whole animal it follows that considerable amounts of amino-sugar can be injected without causing any marked change in the NH_3-N excretion. It is quite likely that in the intact animal the amino-sugars undergo enzymic processes different from those occurring in isolated tissues. The remarkable effect of glucosamine feeding in egg white injury, although transient and probably unspecific, shows that much more study is necessary in the field of amino-sugar metabolism, especially with regard to the nutritional requirements of the organism for these substances. At present no data seem to be available as to whether the amino-sugars can be formed within the organism, or whether they must be added with other exogenous foodstuffs.

Yeast and bacteria act on the amino-sugars both aerobically and anaerobically. The yeast enzyme is rather weak but the enzyme in *B. coli* decomposes amino-sugars very rapidly. The effects of various inhibitors on these enzymes are the same as in animal tissues.

Much attention has recently been given to amino-sugars in connexion with work on the diffusing factor and mucinase, which hydrolyse certain polysaccharides and liberate from them amino-sugars. It is unlikely that the sensitive enzyme system described in this work could play any part or be present in the preparations of testicular diffusing factor, which apart from being stable in the presence of antiseptics like merthiolate, are known to exert their maximum activity at a low *p*H, at which the enzyme causing the oxidative deamination of amino-sugars would no longer be active. In connexion with this it may be interesting to report that bee venom ('Lyovac', Mulford Laboratories) was found to oxidize and deaminate both glucosamine and chondrosamine HCl.

Summary

An enzyme system which decomposes glucosamine HCl, *N*-acetylglucosamine and chondrosamine HCl, was found to exist in animal tissues, yeast and bacteria.

In animal tissues the addition of amino-sugars was found to cause an increase in the O_2 uptake, accompanied by acid formation and production of NH_3. No free CO_2 was formed in the course of the reaction. In yeast and bacteria, the

same processes occur but, in addition, the amino-sugars are attacked also in the absence of O_2.

The enzyme examined is specific for amino-sugars; it bears no relation to either the glucose oxidase or the amino-acid oxidase or the amine oxidase. Its activity was found to depend greatly on the cell structure being preserved. The optimum *p*H was found to lie between 7·4 and 7·8.

From quantitative estimations of the amino-sugar disappearing and the O_2 uptake as well as the NH_3 produced, it appears that the amino-sugars are first converted by oxidation into some product, probably an acid, which in turn undergoes deamination. It was found that glucosaminic acid cannot be regarded as the oxidation product responsible for the NH_3 accumulation. Glucosaminic acid was found to be stable in the presence of animal tissues, yeast and bacteria.

Attempts were made to identify the reaction products. So far, it has been established that there is no formation of lactic, acetic or succinic acid, that substances of ketonic or aldehyde character are absent, and that the products formed from amino-sugars are oxidized by muscle dehydrogenases in the presence of cozymase.

Iodoacetic acid, cinchophen and antiseptics completely abolish the activity of the amino-sugar enzyme. KCN and glyceraldehyde inhibit the oxidation, but have little effect on the NH_3 liberation. Glucose, unlike other sugars, considerably depresses deamination but interferes comparatively slightly with oxidation.

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65. THE METHYLATION OF WOOL WITH METHYL SULPHATE AND METHYL HALIDES

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All the recognized methods of *O*- or *N*-methylation have been applied to proteins. Casein, for example, was boiled with CH_3I and KOH by Skraup & Krause [1909] and later treated with diazomethane by Geake & Nierenstein [1914]. Herzig & Landsteiner [1914] also used diazomethane to methylate casein, edestin and silk fibroin. The same authors [1918] later studied the methylation of wool with diazomethane and after eighteen treatments with this reagent obtained wool with a methoxyl content of 5·89 and 6·28% of CH_3 linked to N. Treatment of wool with methyl alcoholic hydrochloric acid gave a methoxyl content of 3·80 and 1·49% CH_3 as *N*-methyl. Edlbacher [1919] methylated a number of protamines with methyl sulphate and NaOH and determined the number of *N*-methyl but not of *O*-methyl groups. The conclusions drawn from these investigations were rendered doubtful by the discovery of methionine by Mueller [1921]. The *S*-methyl group of this acid yields CH_3I when boiled with HI and thus contributed to the CH_3 content of some of the methylated proteins prepared before 1921. More recently, Felix & Rauch [1931] and Felix & Reindl [1932] have prepared methyl esters of proteins by the action of dry HCl on their suspensions in CH_3OH. They found that the products could be further methylated by either methyl sulphate at *p*H 8 or 9, or by diazomethane. These more fully methylated proteins did not give the Pauly diazo reaction for the free iminazole ring or the Millon reaction for tyrosine. Haurowitz [1938] methylated egg albumin and horse haemoglobin with methyl sulphate. Rutherford *et al.* [1940] have recently made an extensive investigation of the methylation of silk fibroin with diazomethane.

Whilst attempting to methylate wool to obtain evidence of the existence of free thiol groups, we have found that it is possible to introduce CH_3 groups into wool keratin by using methyl sulphate, CH_3I or CH_3Br at ordinary temperatures. Evidence is given below that this reaction is largely, but not entirely, an esterification and appears to take place only when the wool is within its isoelectric region. Some evidence has been obtained that *N*-alkylation occurs at the same time.

Experimental

Unless otherwise stated, the wool used was in the form of a loosely woven flannel, similar to that described in previous investigations [Elsworth & Phillips, 1938].

Determination of CH_3 linked to O and S. All the methylated wools were analysed by Baernstein's method [1932; 1936] in which CH_3 groups linked to O and S, but not to N, are removed as CH_3I. Baernstein [1932] found that eleven different amino-acids and also glucose, when tested by this method, did not yield

any volatile iodide. A similar result was obtained, during the present work, with methylamine. In addition, Barritt [1934] and Bailey [1937], using the method to determine the methionine in wool, were able to show that the sum of the cystine- and methionine-S was almost equal to the total S, suggesting that the method, when applied to wool, does not yield volatile iodide from unexpected sources.

Methylation of wool with methyl sulphate

(*a*) *Influence of* p*H on the reaction.* The wool used in these experiments was in the form of a worsted serge and had been scoured commercially. It was extracted with light petroleum and methylated by shaking for 90 min. with solutions of methyl sulphate in various buffers; 20 ml. of buffer containing 0·2 ml. of methyl sulphate per g. of conditioned[1] wool were used. At the end of each methylation the wool was rinsed in six changes of water, passed through a wringer after each rinse, dried and conditioned. The CH_3 content of the unmethylated wool was 0·06 %. The CH_3 contents of the methylated wools, together with the *p*H of the methylating solutions at the beginning and at the end of each methylation are given in Table 1. The experimental conditions chosen for these methylations were not ideal, since the *p*H of some of the solutions changed too much. The results suggest, however, that the maximum degree of methylation is attained when the *p*H of the methylating solution at the end of the treatment is within the range 2·5–8·5.

Table 1. *The influence of* p*H on the methylation of wool with methyl sulphate*

Buffer	Initial *p*H	Final *p*H	CH_3 %	Total CH_3, less methionine-CH_3 %
0·02*N* H_2SO_4	—	—	0·13	0·07
Phosphate	6·0	2·0	0·29	0·23
,,	7·0	2·5	0·37	0·31
,,	8·0	2·8	0·34	0·28
Borate	9·0	8·1	0·34	0·28
,,	10·0	8·6	0·35	0·29
,,	12·0	7·0	0·37	0·31
Acetate	12·0	9·0	0·13	0·07
,,	12·0	11·8	0·06	0·00

(*b*) *Repeated methylation with methyl sulphate.* The above experiments established optimum *p*H conditions for methylating wool, using a limited amount of methyl sulphate. Repetitions of these treatments were now made to see whether an upper limit existed to the amount of methylation which could be produced in this way. Wool was shaken for 90 min. with phosphate buffer, 0·15*M* with respect to H_3PO_4 (20 ml./g.) and methyl sulphate (0·2 ml./g.). The *p*H of the solution fell during this time from 7·6 to 6·3. A sample of the wool (3–4 g.) was then removed, rinsed with water, as previously described, dried, conditioned and analysed. The remaining wool was methylated again for the same length of time with fresh buffer and methyl sulphate. The CH_3 contents of wool methylated up to twenty times in this manner are plotted against the

[1] All the wools described in this paper as conditioned had been exposed to a controlled atmosphere of 21° and 70 % R.H. until they attained a constant weight. It was found that methylation did not alter the moisture content (14 %), which was reached in this atmosphere at equilibrium. Although all the analyses were made on conditioned wool, the results are recorded as percentages of the anhydrous wool.

number of methylations in Fig. 1. This figure also contains a curve summarizing the results obtained when another sample of wool was treated with methyl sulphate (0·2 ml./g.) and sodium acetate (0·35 g./g. in 15 ml. water), these conditions being somewhat more drastic than when phosphate buffer was used. At 90 min. intervals small samples of wool were withdrawn for analysis and a further 0·2 ml. methyl sulphate and 0·35 g. sodium acetate added to the original

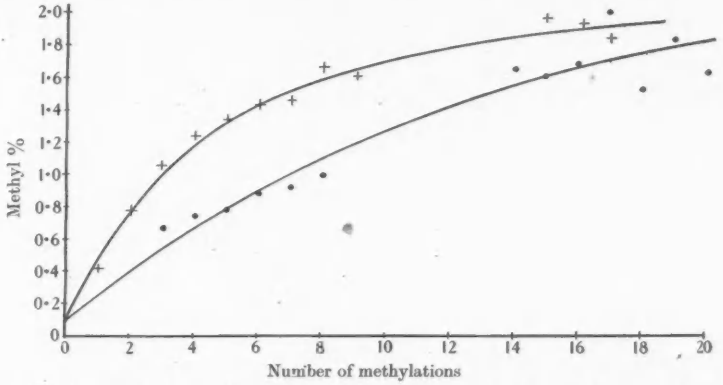


Fig. 1. The methylation of wool with methyl sulphate in phosphate and acetate buffers. +—+ Acetate buffer. •—• Phosphate buffer.

solution. This was repeated nine times and then, after the wool had been washed and dried, for a further eight times. In the phosphate buffer, the initial rate of reaction was fairly rapid; in the acetate buffer, the rate of reaction was more uniform. In both buffers, however, the methylation appears to be reaching a limiting value of 1·9–2·0 % CH_3.

Methylation of wool with methyl iodide and methyl bromide

(*a*) *Methylation of wool with CH_3I.* For reasons that will become apparent later, it was thought that if wool reacted at ordinary temperatures with methyl sulphate it should also react with methyl iodide. Conditioned wool (1 part) was placed in a bottle containing CH_3I (6 parts), and kept at ordinary temperatures in the dark. At intervals samples of the wool were withdrawn, washed with benzene and then extracted with benzene in a Soxhlet apparatus, quinoline being placed in the distillation flask to retain the extracted CH_3I. The CH_3 content of the extracted wool was determined. A portion of the benzene-extracted wool was boiled with water through which steam was passed for 1 hr., dried, conditioned, and the CH_3 content redetermined. Another portion of the benzene-extracted wool (1·5 g.) was shaken with phosphate buffer (*p*H 6·0, 75 ml.) for $3\frac{1}{2}$ hr., rinsed in water, dried, conditioned and analysed. In Table 2, the CH_3 contents of wools that had been methylated with CH_3I for increasing periods of time and then extracted with benzene are recorded, together with the CH_3 contents of the extracted wools after distillation in steam and also after extraction with phosphate buffer. From these results it is apparent that after the methylated wool has been extracted with benzene, subsequent distillation in steam or extraction with phosphate buffer causes only small decreases in its CH_3 content.

Table 2. *Percentage CH_3 content of wool attained at ordinary temperatures with CH_3I*

Time of exposure to CH_3I, hr. ...	2	6	17	24	90	144	336
(i) After extraction with benzene	0·38	0·30	0·38	0·43	0·53	0·41	0·45
(ii) After extraction with benzene followed by steam distillation for 1 hr.	0·36	0·24	—	0·36	0·49	0·35	0·44
(iii) After extraction with benzene followed by extraction with buffer *p*H 6·0	0·36	0·23	0·36	0·39	0·52	0·40	—

It appears that wool reacts fairly rapidly with CH_3I, and that prolonged periods of exposure cause only a slight further reaction. The irregular variation in the analyses after different times of exposure is most likely due to slight variation in the samples of wool taken for each experiment, and the CH_3 figures after, say, 2 days may be taken to be sensibly constant. Another irregularity that has been observed is that the rate of reaction may vary within wide limits. For example, another sample of wool brought to equilibrium with a phosphate buffer of *p*H 5·9, and then dried and conditioned, at first reacted much more slowly; after 2 hr. exposure to CH_3I its CH_3 content was 0·09 %, after 24 hr. 0·19 %, but after 144 hr. 0·45 %. The reaction appears to depend on the physico-chemical condition of the wool. Thus when conditioned wool was heated under reflux with CH_3I for 2 hr., it contained 0·30 % CH_3 after benzene extraction. On the other hand, when the CH_3I was diluted with an equal volume of ether, and the wool heated under reflux for 2 hr., its CH_3 content, after benzene extraction, was 0·14 %. Dried wool reacted with CH_3I very slowly; after 72 hr. exposure, the wool contained only 0·09 %, i.e. only slightly more than that due to methionine (0·06 %).

Presumably when wool is methylated with CH_3I, for each CH_3 group introduced, one atom of anionic iodine is liberated. The determination of this anionic iodine thus provides an additional measure of the degree of methylation that occurs. The samples (1·5 g.) of methylated and benzene-extracted wools, the analyses of which are given in Table 2, were shaken for 3½ hr. with 75 ml. of buffer, *p*H 6·0. These conditions were chosen as the result of separate experiments which showed that this method gave the maximum extraction of iodide. The filtered buffer and washings from the wool were acidified with dilute HNO_3, the iodide precipitated with silver nitrate and the excess silver nitrate determined with potassium thiocyanate. The results are recorded in Table 3, in which CH_3 equivalent to the iodide is also given. By subtracting the methionine-CH_3 (0·06 %) from the figures given for the total CH_3 in Table 2, the figure for the total *O*-methyl is obtained. This is seen to be less than the CH_3 equivalent of the anionic iodide found. The extra methylation that occurs, as revealed by the

Table 3. *Anionic iodine contents of wool exposed to CH_3I for increasing periods of time and then extracted with benzene*

Time of exposure hr.	Anionic iodine %	CH_3, equivalent to anionic I found %	Observed CH_3, less methionine-CH_3	Excess CH_3 (calc.) %
2	3·68	0·43	0·32	0·11
6	3·46	0·41	0·24	0·17
17	3·83	0·45	0·32	0·13
24	3·98	0·47	0·37	0·10
90	5·30	0·63	0·47	0·16
144	3·83	0·45	0·35	0·10

iodide figures, probably represents *N*-methylation which is not included in the CH_3 as determined by the Baernstein method. This excess CH_3 is given in the fifth column of Table 3.

(*b*) *Repeated methylation of wool with CH_3I.* The wool (30 g.) was exposed to CH_3I, as in the previous experiments, except that the operation was repeated four times at intervals, recorded in Table 4. Before each exposure, the wool was buffered to *p*H 7·9 with borate buffer.

Table 4. *The CH_3 contents of wool after repeated exposures to CH_3I*

Time of exposure days	CH_3 %	I %
14	0·52	4·77
28	0·80	—
46	1·02	—
88	1·06	—

After hydrolysis for 24 hr. with 5*N* HCl and evaporation *in vacuo*, the CH_3 content of the hydrolysate was 0·10 %.

(*c*) *Methylation of wool with CH_3Br.* Conditioned wool (1 part), buffered to *p*H 7·7 in borate, was treated with CH_3Br (1·5 parts) in a tightly stoppered bottle at room temperature. At intervals, the pressure was released, the CH_3Br allowed to evaporate and the wool conditioned and analysed. The results obtained are given in Table 5.

When wool, methylated in this manner and having 1·30 % CH_3, was subjected to complete hydrolysis with HCl, the CH_3 content of the hydrolysate was 0·08 %. It is noteworthy that the 'excess' CH_3 introduced by the action of CH_3Br is greater than that obtained using CH_3I.

Table 5. *Methylation of wool with CH_3Br*

Total time of exposure, days	CH_3 %	Br %	'Excess' CH_3 (calc.), %
1	0·29	—	—
4 (CH_3Br renewed)	0·58	4·98	0·42
6	0·65	—	—
18 (CH_3Br renewed)	0·92	7·53	0·55
44 (Re-buffered and CH_3Br renewed)	1·30	—	—

(*d*) *Methylation of silk.* Because of the difference in chemical constitution between wool and silk, it was of interest to study the methylation of silk in a

Table 6. *Methylation of silk*

(*a*) With methyl sulphate (phosphate buffer).

No. of methylations	CH_3, %
2	0·20
5	0·40
9	0·45
11	0·46
13	0·50
14	0·45

(*b*) With CH_3I

Time of exposure days	CH_3 %	I %	CH_3, equivalent to I found %	'Excess' CH_3 (calc.) %
19	0·37	4·72	0·56	0·19

similar manner. Silk, in the form of an unweighted fabric, was methylated with methyl sulphate in a phosphate buffer, and with CH_3I, using the conditions previously described for wool. The results are given in Table 6.

Stability of the methoxyl groups introduced

The CH_3 content found by analysis after these treatments might conceivably have been due to retention of the methylating reagent in the wool without any definite chemical reaction having occurred. Experiments were made to determine whether the CH_3 content could be reduced by certain mild treatments.

(i) After heating in a strong stream of air at 115° for 2 hr., samples of wool methylated with methyl sulphate and containing 0·30 and 1·62 % CH_3 were found to give unchanged analyses, although the same treatment for 10 min. would remove 14 % of water from conditioned wool. Further, wool methylated with CH_3I and then extracted with benzene contained 0·30 % CH_3 after heating in a current of air at 115° for 30 min. and 0·29 % after 60 min.

(ii) In order to obtain evidence of the degree of resistance to hydrolysis of the methyl groups which had been introduced, the wool, methylated with methyl sulphate, was steam distilled. Wool (2 g.) containing 0·36 % CH_3 was covered with water (20 ml. *p*H 8) and steam was blown into the water for the times given in Table 7. At the end of each distillation, a sample of the wool was dried, conditioned and then analysed. The analyses show that the CH_3 is held tenaciously by the wool, although it is gradually lost as the time of distillation increases. The results of a similar series of experiments on methylated wools of higher CH_3 contents are also given in Table 7. In these experiments, before the steam was blown into the flask, the methylated wool was covered with 5 ml. of a borate buffer (*p*H 7) and 15 ml. water, in order to prevent a large alteration in *p*H during the distillation. A rapid drop in the CH_3 content of these wools is observed at the beginning of the distillation, followed by a smaller drop as distillation is continued. For a 'fully methylated' wool, the CH_3 groups introduced above a certain value (about 0·6–0·7 % CH_3) are much more readily removed by distillation than those below this value. This may indicate that there are two centres which are being methylated, from one of which the CH_3 groups are readily removed, whilst the CH_3 groups attached to the other resist hydrolysis under the conditions employed. This point is discussed more fully later.

Table 7. *The effect of exposing wool, methylated with methyl sulphate, to a current of steam*

CH_3, %	Duration of steam distillation, min.								
	0	5	10	20	40	60	120	180	240
	—	0·28	0·28	0·24	0·23	0·27	0·22	—	0·16
	0·31	—	—	—	—	0·23	0·20	0·19	0·16
	0·66	—	—	—	—	0·49	0·39	0·38	0·31
	1·01	—	—	—	—	0·64	0·59	0·58	0·46
	1·07	—	—	—	—	0·75	0·67	0·65	0·58
	1·63	—	—	—	—	0·72	0·71	0·57	0·55

(iii) When wool (CH_3, 0·37 %) that had been methylated once with methyl sulphate and buffer was placed in a 0·5 % solution of sodium sesquicarbonate (*p*H 10·0) or in 0·1 *N* NaOH, and steam was blown through the water, the CH_3 was quickly removed and in 30 min. the CH_3 content of the wool returned to its pre-methylation value of 0·06 %. This experiment provided clear evidence that the CH_3 was linked to the wool by a readily hydrolysable linkage and was

not, therefore, attached either to the phenolic group of tyrosine or to the S of cysteine.

Similar results were obtained with more highly methylated wools. A wool containing 1·62 % CH_3 gave a value of 0·21 % CH_3 after distillation with 1 % sodium sesquicarbonate (*p*H 10) for 1 hr. When the wool was distilled in a current of steam for 1 hr. and then treated with sodium sesquicarbonate, the final CH_3 content was 0·10 %. It is evident that even after repeated treatment with methyl sulphate the number of methoxyl groups which are attached to the wool by non-hydrolysable linkages is extremely small. When the wool was subjected to complete hydrolysis (24 hr.) with HCl, the CH_3 content of the hydrolysate, determined after evaporation *in vacuo*, corresponded to the methionine content of the original wool.

(iv) Experiments were made to determine the ease with which the CH_3 of methylated wool could be removed by extraction with solutions of different *p*H. Samples of wool (2·0 g., CH_3, 1·66 %) methylated with methyl sulphate and buffer were treated with 100 ml. of 'Universal' buffer of different *p*H (containing acetic, phosphoric and boric acids and NaOH) at room temperature in closed bottles. After standing overnight, the wools were washed once with water, dried, conditioned and analysed. The *p*H of the solutions from which the wools had been taken was determined with a glass electrode. The results are recorded in Table 8.

Table 8. *The effect of extracting methylated wool with buffers of different* p*H*

Initial *p*H	0·46	1·8	2·82	4·59	6·27	8·38	9·46	10·45
Final *p*H	0·75	1·86	2·99	4·64	6·32	8·10	9·03	9·44
Residual CH_3, %	1·09	1·44	1·40	1·32	1·24	1·15	0·82	0·87

Titration curve of wool after methylation

(*a*) *Wool methylated with methyl sulphate.* A large sample of wool fabric was methylated in buffer at *p*H 6 and on analysis contained 0·32 % CH_3 and 0·21 % after steam distillation for 60 min. The wool was remethylated and then contained 0·30 % CH_3 after distillation in steam. The disulphide contents of the wool before and after methylation were 2·93 and 2·88 %, respectively. Samples of the methylated and unmethylated wool fabrics were washed with distilled water and finally brought to equilibrium with water at *p*H 5. Each fabric was cut up into about 150 pieces, and samples (1–2 g.) of each weighed and put into bottles containing either solutions of H_2SO_4 or NaOH in which they were kept with occasional shaking for 6 days. The *p*H of the initial and final solutions was determined with the glass electrode. The more concentrated solutions were titrated, using bromocresol purple as indicator. Some of the more alkaline solutions developed an odour resembling that of the methylamines. The results obtained are plotted as ml. *N* acid or alkali absorbed by 100 g. of anhydrous wool in Fig. 2.

In order to detect any possible alteration in the water-holding capacity of the wool caused by methylation, which would have affected the calculations used to construct the curves of Fig. 2, samples of the wools were squeezed, dried at 65°, and then conditioned. The moisture content of each sample was determined by the method of Barritt & King [1926] by drying in a slow current of air at 110°. No appreciable difference was found between the moisture contents of the methylated and unmethylated wools.

(*b*) *Wool methylated with methyl iodide.* A further sample of wool, after buffering to *p*H 8 in borate buffer, was treated with CH_3I. After 11 days the wool was extracted with benzene and found to contain 0·59 % CH_3. The methylated and unmethylated wools were washed with distilled water, and brought into equilibrium with water at *p*H 5, the subsequent procedure being as described in (*a*) above. The results are plotted in Fig. 2.

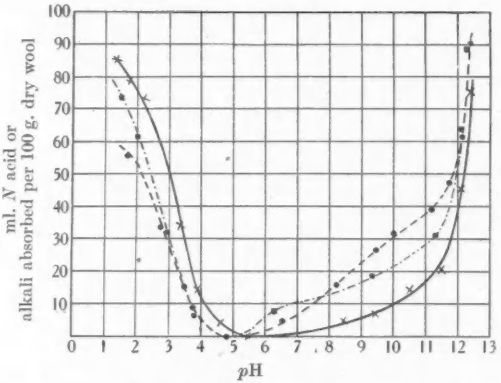


Fig. 2. The titration curves of wool before and after methylation with methyl sulphate and with methyl iodide. ×—× Untreated. •– –• Methylated with CH_3I (0·59 % CH_3). ■—·—■ Methylated with methyl sulphate (0·25 % CH_3).

In agreement with the hypothesis that the wool is esterified by both methylating agents, less acid is required to suppress the ionization of the carboxyl groups of the wool after methylation, being equivalent, at *p*H 1·5, to 11·3 ml. *N* acid (i.e. 0·17 % CH_3) in the wool methylated with methyl sulphate, and to 23 ml. *N* acid (i.e. 0·32 % CH_3) in the wool methylated with methyl iodide. No quantitative conclusions can, however, be drawn from these curves since some of the CH_3 may have been lost when the methylated wools were purified. On the alkaline side of *p*H 5, it is important to notice that the two methylated wools behave differently. Although both wools neutralize an appreciable amount of alkali from *p*H 5 to 7, the wool methylated with methyl sulphate neutralizes more than the wool methylated with CH_3I. This alkali is neutralized during the back-titration of some feebly basic group. In the more alkaline solutions, the wool methylated with CH_3I neutralizes more alkali than the wool methylated with methyl sulphate. It is probable that this alkali is neutralized during the hydrolysis of carbomethoxyl groups. On this basis, whereas methylation with CH_3I favours esterification of carboxyl groups, methylation with methyl sulphate favours the production of the feebly basic groups.

Discussion

There can be little doubt that the CH_3 groups found in the methylated wools described are chemically combined, since they persist after the wool has been extracted with benzene and water, subjected to steam distillation and exposed to a current of hot air. The clearest indication of their state of combination is given by the ease with which they can be removed from the wool by acid hydrolysis and by boiling it with buffer solutions higher than *p*H 9. In addition, when

methylated wools are steam distilled in the presence of alkali, methyl alcohol can be detected in the distillate by Schryver & Wood's method [1920]. The removal of all the CH_3, with the exception of the methionine-CH_3, by this method indicates clearly that the added CH_3 is not derived from methylated thiol groups or from methylated tyrosine. Further, since the method used in the determinations of CH_3 is unlikely to return CH_3 attached to nitrogen, the CH_3 is not derived from methylimide groups. Some *N*-methylation occurs, however, when wool is methylated with CH_3I and CH_3Br, since the amount of anionic halide introduced is more than equivalent to the CH_3 as estimated by the Baernstein method.

When *O*-methylation takes place, the most probable reaction is the esterification of carboxyl groups. This conclusion is of particular interest since carboxylic acids are not esterified readily under the conditions used in the present experiments, by either methyl sulphate or methyl iodide. On the other hand, both these substances esterify the zwitterions of amino-acids [Taylor & Baker, 1937].

$$NH_3^+CH_2COO^- \xrightarrow{MeI} NH_3^+CH_2COOMe\ [I^-].$$

Engeland [1910], for example, found that phenylalanine was methylated on boiling with methyl alcoholic KOH and CH_3I, and Novak [1912] employed methyl sulphate and NaOH with a variety of amino-acids.

Free amino-acids do not, however, exist in wool in any appreciable quantities and therefore the methyl sulphate, iodide and bromide must esterify combined amino-acids. If these acids are monoaminomonocarboxy-acids, they may terminate the main polypeptide chains to which they must be linked through their amino groups. More probably they are monoaminodicarboxy-acids which are built into the polypeptide chain and esterify because their carboxyl groups terminating the side-chains form zwitterions with the amino groups terminating the side-chains derived from the monocarboxydiamino-acids. In other words, esterification occurs at salt linkages. Evidence for the existence of linkages of this nature in keratin has been accumulated by Speakman & Hirst [1931; 1933].

There are several indications that wool keratin will only esterify when within its isoelectric region. For example, the data given in Table 1 show that the highest degree of methylation was obtained when wool was methylated with solutions of methyl sulphate of *p*H 2·5–8·5. Further, anhydrous wool did not methylate during 75 hr. exposure to CH_3I, nor would conditioned wool methylate when boiled with an ethereal solution of CH_3I, although it underwent limited methylation when boiled with CH_3I. Moreover, when wool with a high CH_3 content is extracted with solutions of different *p*H, the CH_3 groups show the greatest stability in the range *p*H 2–8, solutions with *p*H values outside this range causing a much larger fall in the CH_3 content (Table 8).

The hypothesis that methylation of carboxyl groups occurs owing to the influence of the adjacent basic groups of histidine, lysine and arginine seems to be qualitatively adequate to explain the results already obtained. Amino-acid analyses of wool keratin are available from which it is possible to see the extent of the quantitative agreement between the CH_3 content of methylated wool and the CH_3 content that might be expected. In Table 9 the percentages of the amino-acids in wool likely, on the above hypothesis, to be involved in zwitterion formation are given, together with the equivalent amount of CH_3 to which they might give rise assuming that they form salt linkages.

The CH_3 content of wool should reach a maximum value equal to the free carboxyl groups, plus the CH_3 due to the *S*-methyl of the methionine present and to its probable conversion into a sulphonium group. The free carboxyl

Table 9. *The composition of wool*

Amino-acid		% of wool	Equivalent CH_3, % of wool
Arginine	Vickery [1940]	10·4	0·90
	Marston [1928]	10·2	0·88
Lysine	Vickery & Block [1930]	2·3	0·24
	Block [1939]	2·5	0·26
	Marston [1928]	2·8	0·29
Histidine	Vickery & Block [1930]	0·7	0·07
Glutamic acid	Abderhalden & Voitinovici [1907]	12·9	1·32
	Speakman & Townend [1937]	15·3	1·56
Aspartic acid	Abderhalden & Voitinovici [1907]	2·3	0·26
	Speakman & Townend [1937]	7·3	0·82
Methionine	This paper	0·6	0·12*
Amide-N	Speakman & Townend [1937]	1·37	1·47

* Assuming methionine combines with the methylating agent to give a sulphonium compound.

groups of wool are limited to the carboxyl groups of glutamic and aspartic acids which are not linked in the main chain or combined with ammonia as amide groups. Using the figures of Abderhalden & Voitinovici [1907] for glutamic and aspartic acids and Speakman & Townend's [1937] figure for amide-N, the CH_3 content of fully methylated wool might have a possible value of $1{\cdot}58-1{\cdot}47+0{\cdot}06=0{\cdot}17\,\%$. If the later figures of Speakman & Townend for glutamic and aspartic acids are utilized, the CH_3 content would be $0{\cdot}96\,\%$. Assuming that CH_3I (or sulphate) adds on to the sulphur atom of methionine [Toennies, 1940, 1, 2], these values would be increased by $0{\cdot}06\,\%$. The number of CH_3 groups that wool can take up is much larger than even the higher of these figures.

It would seem to be improbable that appreciable hydrolysis of the peptide links of the main chains, liberating further carboxyl groups which might then undergo methylation, would occur during even prolonged treatment at the *p*H employed in these experiments. Moreover, hydrolysis of this type could hardly occur during treatment of wool with CH_3I or CH_3Br, and yet one wool methylated with CH_3Br contained more CH_3 ($1{\cdot}30\,\%$) than could be accommodated by the free carboxyl groups.

The results obtained on methylating silk fibroin are of importance in this connexion. It can be seen from Table 6 that when the methods used for wool are applied to silk a maximum value of $0{\cdot}45\,\%$ of CH_3 is reached. The greater part of this CH_3 is removed on treatment with alkali. Rutherford *et al.* [1940] have recently obtained similar results after methylating silk with diazomethane. These authors found that the free phenolic groups of tyrosine were readily methylated, but in addition the total methoxyl content was found to include some 'extra' methoxyl groups, corresponding to 0·26 millimols/g. (or $0{\cdot}39\,\%$ CH_3) which were removed as CH_3OH on alkaline hydrolysis and were not attached to primary amino or serine hydroxyl groups. These authors tentatively assumed that the 'extra' groups were located on carboxyl groups. Since, as far as we are aware, no one has succeeded in isolating dicarboxylic amino-acids from silk fibroin, the amounts of dicarboxylic acids present in silk are likely to be extremely small. The diamino-acid content of silk is also very low: Vickery & Block [1931] found $0{\cdot}74\,\%$ arginine, $0{\cdot}25\,\%$ lysine and $0{\cdot}076\,\%$ histidine. Salt linkage formation between the basic side-chains of these acids and free carboxyl groups derived from other acids could therefore only give rise to zwitterions capable of being combined with $0{\cdot}10\,\%$ CH_3.

The esterification of carboxyl groups cannot account for all the hydrolysable CH_3 with which wools and silk will combine. In methylated wool as much as 0·7 %, and in methylated silk as much as 0·4 % CH_3, are probably linked to oxygen atoms which are not present in carboxyl groups. We are therefore forced to entertain the possibility that the oxygen atoms to which this CH_3 is attached are the oxygen atoms of the peptide linkages. Several workers have discussed the possibility of an enolization in peptides and proteins, which can theoretically occur in two ways:

$$H_2N.CH(R_1).CO.NH.CH(R_2).COOH \rightleftarrows H_2N.C(R_1)(OH)=C(R_2)-NH.CH.COOH$$

$$H_2N.CH(R_1).CO.NH.CH(R_2).COOH \rightleftarrows H_2N.CH(R_1).C(OH)=N.CH(R_2).COOH$$

Enolization of the first type involving loss of asymmetry in the carbon atom was postulated by Dakin [1912–13] to explain the racemization of certain amino-acids in proteins by the action of alkali at low temperatures. The idea was later elaborated by Levene and co-workers [Levene *et al.* 1927; Levene *et al.* 1931; Levene & Yang, 1933] in a study of the racemization and hydrolysis of peptides under the influence of alkali. Levene also expressed the opinion that enolization of the second type, involving the nitrogen atom, could occur in polypeptides and proteins.

Moreover, it is possible to obtain stable isomers of compounds containing the peptide linkage. This isomerism has been shown to be due to enolization. Thus Leuchs & Manasse [1907] demonstrated the existence of two isomeric forms of glycylglycine carboxylic acid, the 'α' form having the lactam formula, the 'β' having the lactim formula:

$$HOOC.NH.CH_2.CONH.CH_2.COOH \qquad \alpha$$
$$HOOC.NH.CH_2.C(OH)=N.CH_2.COOH \qquad \beta$$

In accordance with expectation, when the enolizable hydrogen atom is replaced by a phenyl group, only the 'α' form is obtained. Leuchs & La Forge [1908] have prepared similar isomers of *N*-carboxydiglycylglycine.

If one or more of the peptide links in wool or silk can readily enolize, methylation might be expected to occur. It is not, however, necessary to assume that the imido-hydrogen atom migrates. More probably the methylating agent adds on to a peptide linkage which has become a resonance hybrid of the two structures (I) and (II) and as such, since it approaches a zwitterion in constitution, might conceivably methylate in a similar manner.

(I) $-C(=O)-N(H)-$ ⟷ (II) $-C(-O^-)=N^+(H)-$ $\xrightarrow{MeI}$ $-C(=O)-N^+(Me)(H)-\ I^-$ (III)

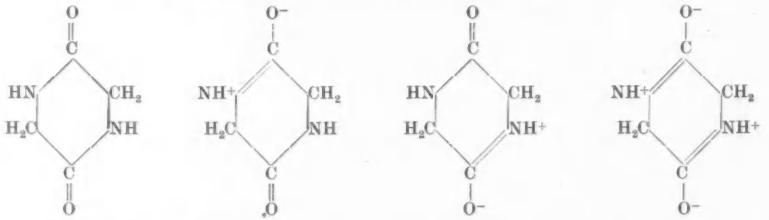
(IV) $NH_2-C(=O)-NH$ ⟷ (V) $NH_2-C(-O^-)=NH^+$

In support of this view, urea which undergoes methylation on the oxygen atom when treated with methyl sulphate has been considered to be a resonance-hybrid of the two forms (IV) and (V) [Taylor & Baker, 1937].

On the chemical side, two pieces of evidence are in agreement with the presence of methylated peptide linkages possibly possessing the structure indicated in (III). The first is the lability of some of the CH_3 in the highly methylated wools, and secondly, the neutralization of alkali by methylated wools

from *p*H 5 to 7 (Fig. 2). An imido-group, which had undergone salt formation as indicated in (III), would be expected to hydrolyse freely and to lose HI within this *p*H range.

Physical evidence supporting the possible occurrence of 'activated' peptide linkages is also available. From the results of an X-ray examination of the crystal structure of diketopiperazine, Corey [1938] concluded that the diketopiperazine molecule was a resonance hybrid of four possible structures, three of which contain one or more zwitterionic peptide linkages.



Ellis & Bath [1939] found that the infra-red absorption spectrum of diketopiperazine also supported Corey's idea of electron resonance. Karrer *et al.* [1923] obtained a derivative of a tautomeric form of diketopiperazine in which the hydrogen atom is enolized in the above sense. These authors found that when the silver derivative of diketopiperazine reacted with benzyl chloride, a benzyl group was attached to the oxygen atom of the peptide bond, 2:5-dihydroxydihydropyrazine dibenzyl ether being produced. This compound was readily hydrolysed by dilute acids to benzyl alcohol and glycine. Methylation would not be expected to occur at all the peptide linkages in wool and silk, but only at certain specific links which can assume the appropriate electronic state, or, some state approaching it electronically, due to the presence of adjacent activating groups.

The total CH_3 content of methylated wool may thus include two types of *O*-methyl groups: one attached to the free carboxyl groups of glutamic and aspartic acids, the other attached to peptide linkages. On this hypothesis, a large proportion of the CH_3 groups in methylated silk would be attached to peptide linkages. Further work is in progress to enable a clearer distinction to be drawn between methylation at these two centres.

Summary

1. Wool can be methylated with methyl sulphate, iodide and bromide, at ordinary temperatures.

2. With each reagent, both *O*- and *N*-methylation takes place.

3. The experimental results indicate that the *O*-methylation consists partly of an esterification of the free carboxyl groups.

4. Evidence is presented which suggests that *O*-methylation also takes place at peptide linkages.

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66. THE ISOLATION OF AMINO-ACIDS FROM RUBBER LATEX

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After an examination of the chemical composition of the 'resin' of rubber had been carried through [Whitby *et al.* 1926] there was initiated a chemical examination of the aqueous serum left on separating rubber from the latex of *Hevea brasiliensis*. This latter was carried as far as a study of the amino-acids present, and had then to be interrupted. As it has not been possible since to resume it, and as several papers on the amino-acid composition of the proteins of *Hevea* latex have recently appeared, it is now thought desirable to put on record the results which were obtained.

While several investigations, mentioned below, have been made of the amino-acids derived by hydrolysis from the proteins of rubber and latex serum, only one previously published investigation has been concerned with the amino-acids present as such in latex. McGavack & Rumbold [1934] isolated *d*-alanine from latex serum and secured some evidence of the presence of phenylalanine. In the present investigation the following seven amino-acids were isolated from the serum: tyrosine, *l*-leucine, *d*-*iso*leucine, *d*-valine, *d*-arginine, *l*-aspartic acid and *i*-proline. Indications were also obtained of the presence of phenylalanine.

It may be noted that one of these, *d*-valine, was previously isolated from the acetone extract of rubber (sheet form) [Whitby *et al.* 1926]. Clearly, it had been taken up by the coagulum from the serum and had escaped complete removal in the preparation of the rubber.

It is interesting to compare these findings as to the free amino-acids present in *Hevea* latex with the results of the examination by various investigators of the amino-acids obtained by the hydrolysis of protein from rubber and serum. Belgrave [1925, 1] obtained evidence of the presence of glycine and proline in proteins obtained (*a*) from latex serum by precipitation with alcohol, (*b*) from rubber by exhaustive extraction with benzene. Later work by Belgrave [1925, 2] on the former protein indicated the presence, in addition, of arginine (in high proportion), cystine, histidine and lysine. Midgley *et al.* [1937] obtained the following from protein isolated from rubber: glycine, proline, arginine, histidine, lysine, leucine, aspartic acid and a representative of the group comprising alanine, phenylalanine, hydroxproline and serine. They state that the following were definitely absent: cystine, glutamic acid and tyrosine. Subsequent authors, however, working it is true with protein isolated from rubber or latex in different ways, have identified these three amino-acids in hydrolysates.

In protein obtained from latex serum by precipitation with ammonium sulphate, Altman [1935] identified and estimated proline, arginine, histidine, leucine, aspartic acid, alanine, dihydroxyphenylalanine, tyrosine, glutamic acid, hydroxyproline and valine. Tristram [1940] analysed protein obtained by extraction of dried, fresh latex for the following nine amino-acids, all of which were present and which in total constituted about 50% of the protein: arginine, histidine, leucine, aspartic acid, tyrosine, glutamic acid, cystine, methionine and tryptophan.

Table 1. *Amino-acids in latex and from latex proteins*

Proteins isolated by
1. Precipitation of serum with alcohol.
2. Precipitation of serum with ammonium sulphate.
3. Heat coagulation of serum.
4. Extraction of dried latex.
5. Separation from rubber.
6. Separation from rubber.

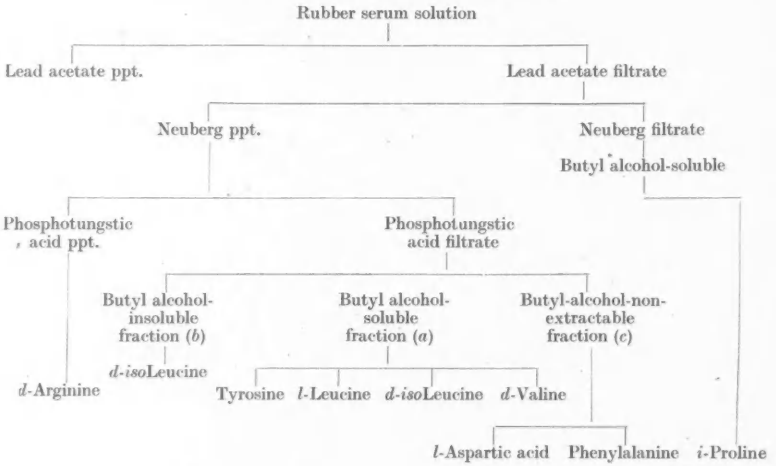
In latex serum		By hydrolysis of proteins					
		1		3	4	5	6
McGavack & Rumbold [1934]	Present authors	Belgrave [1925, 1, 2]	2 Altman [1935]	Present authors	Tristram [1940]	Belgrave [1925, 1]	Midgley *et al.* [1937]
—	—	Glycine	—	Glycine	—	Glycine	Glycine
Alanine	—	—	Alanine	—	—	—	—
—	Valine	—	Valine	—	—	—	—
—	Leucine	—	Leucine	—	Leucine	—	Leucine
—	*iso*Leucine	—	—	—	—	—	—
Phenylalanine	Phenylalanine	—	Dihydroxyphenylalanine	—	—	—	—
—	Tyrosine	—	Tyrosine	Tyrosine	Tyrosine	—	—
—	Aspartic acid	—	Aspartic acid	Aspartic acid	Aspartic acid	—	Aspartic acid
—	—	—	Glutamic acid	Glutamic acid	Glutamic acid	—	—
—	Arginine	Arginine	Arginine	Arginine	Arginine	—	Arginine
—	—	Histidine	Histidine	Histidine	Histidine	—	Histidine
—	—	Lysine	—	Lysine	—	—	Lysine
—	Proline	Proline	Proline	Proline	—	Proline	Proline
—	—	—	Hydroxyproline	—	Tryptophan	—	—
—	—	Cystine	—	—	Cystine	—	—
—	—	—	—	—	Methionine	—	—

In the present work a cursory examination of the heat-coagulable protein of latex serum led to the isolation from its hydrolysate of glycine, proline, aspartic acid, glutamic acid and tyrosine, and gave evidence of the presence, in addition, of histidine, arginine and lysine.

Table 1 summarizes the results of investigations made to date on the identification, isolation and estimation of the amino-acids of rubber, latex and latex serum.

In isolating, in the present investigation, amino-acids from latex serum, the method of fractionation employed was essentially that of Vickery [1924; 1925] and of Vickery & Leavenworth [1925], in which use is made of the mercuric acetate-sodium carbonate reagent of Neuberg & Kerb [1912] to precipitate the amino-acids as their carbonates and the purines as mercury complexes. The chief modification introduced was in the separation of the monoamino-acids and proline from the bases and dicarboxylic amino-acids. For this separation use was made of the butyl alcohol extraction procedure of Dakin [1918; 1920] which has proved so successful when applied by that worker to acid hydrolysates of proteins. The results of applying this procedure to the present case, however, were in keeping with the experience of Hunter [1925], who found that a sharp separation could not be obtained with enzymic digests of proteins containing peptide material. It is indeed hardly surprising that the butyl alcohol extraction method did not follow the same course and was not as complete and clear cut with the present material as with a protein hydrolysate, because of the presence, in addition to amino-acids, of a host of other organic and inorganic compounds. There is, for example, little doubt that acid-hydrolysable peptides were present, as Vickery has shown to be the case with alfalfa juice. Nevertheless, in spite of these circumstances, the method was of value and made it possible to isolate and identify tyrosine, *l*-leucine, *d-iso*leucine, *d*-valine, *d*-arginine, *i*-proline. The general scheme of fractionation is outlined in Table 2.

Table 2. *Outline of fractionation procedure*



Every attempt to arrive at the composition of a complex organic mixture of biological origin such as a solution of chemical entities constituting a plant juice

requires in the first instance much preliminary pioneering work to determine what substances and groups of substances are actually present. In the initial stages of the present study an attempt was made to keep an audit of the nitrogen separation, but many of the fractions obtained proved on further investigation to be so intractable that this had soon to be abandoned.

Experimental

A sample of serum solids, supplied by the Central Rubber Station, Buitenzorg, Java, and prepared by evaporating serum obtained by the coagulation of fresh latex with acetic acid, was used in some preliminary experiments. It was found that, although originally completely water-soluble, an appreciable amount had become insoluble during evaporation and storage. This insoluble material was isolated by filtration, washed with hot water and dried. The resulting hard, dark brown material had no melting point and was found to be protein in nature with 2·15 % ash. Repeated solution in NaOH and precipitation with acid gave a fairly white product. It will thus be seen that originally this protein was a normal soluble constituent of rubber latex, and that, while it was not precipitated by acetic acid (in the cold) during the coagulation of latex, subsequent heating had denatured it.

According to the view of Belgrave [1925] and of Belgrave & Bishop [1923] the non-protein nitrogenous compounds in latex are destined for the synthesis of proteins or are their decomposition products. If this be true then an analysis of the protein for constituent amino-acids should give helpful information as to nitrogenous substances which might be expected to occur in the free state in latex serum. With this object in view a cursory examination of the protein was made.

The protein (100 g.) was hydrolysed with six times its weight of H_2SO_4 (38 %) for 24 hr. The mineral acid was removed by adding baryta to *p*H 3 and the solution of amino-acids was concentrated to 350 ml., when tyrosine began to crystallize out. The solution was then brought to *p*H 5·5 with KOH and concentrated to 200 ml. After 18 hr. at 0° the separated material was filtered off. On repeated crystallization from hot water, this gave 2·5 g. of pure tyrosine.

The concentrated solution of amino-acids was then subjected to a continuous butyl alcohol extraction [Dakin, 1918; 1920]. The fraction separating from the butyl alcohol (monoamino-monocarboxylic acids, aliphatic and aromatic) weighed 31 g. From this mixture glycine was isolated as the ethyl ester hydrochloride: M.P. 144°.

The fraction soluble in butyl alcohol (proline and hydroxyproline) weighed 5·14 g. Some difficulty was experienced in isolating proline as hydantoin but evidences of its presence were found. The fraction not extracted by butyl alcohol was, after removal of the solvent, passed through the Foreman [1914] procedure for the separation of the dicarboxylic acids. In this way 11·4 g. of copper aspartate and 1·75 g. of glutamic acid hydrochloride (M.P. 196–197°) were obtained. The base fraction obtained from the Foreman filtrate by precipitation with phosphotungstic acid was examined by the Kossel procedure as given by Plimmer [1917] and although, for various reasons, the analysis was not completed, evidence of the presence of arginine, histidine and lysine was obtained.

From the cursory examination of the protein described above it will be seen that it is quite similar to most plant proteins, and, on the hypothesis of Belgrave, gives hints as to the possible amino-acids present in the serum of *Hevea brasiliensis*.

The actual work on serum solids was done on a quantity of serum solution (kindly furnished by the Naugatuck Chemical Company) obtained from latex preserved with ammonia and containing sodium alginate, which had been used to cause separation of the rubber by creaming. An attempt was made to evaporate this serum solution *in vacuo*, but the presence of sodium alginate caused violent frothing and it was necessary to concentrate from 55 gallons to 10 l. at atmospheric pressure and 75° with the aid of constant stirring and an electric fan to carry off the vapour. The time taken was 74 hr., and during the concentration about 2 kg. of rubber separated in small flocks, which were removed, together with coagulated protein, by skimming. Towards the end of the operation magnesium oxide was added to remove ammonia. Finally, the cooled concentrate was diluted with alcohol to precipitate the residual rubber and protein, together with the major part of the alginic acid, which could then be filtered off. To the filtrate were added 1200 g. normal lead acetate to remove proteoses and inorganic acids having insoluble lead salts and also to help clarify the solution under investigation. Not much precipitation occurred, however, until the solution was diluted with water. The precipitate was filtered, washed and the filtrate and washings saturated with H_2S to remove lead. The clarified solution was then concentrated *in vacuo* at 30° in the presence of charcoal, filtered and made up to a volume of 10 l. Of this 3800 ml. were taken for further investigation, and the results of a preliminary analysis by standard methods are given in Table 3.

Table 3

	g. in 3800 ml.	% of total N
Total N	124·6	100
Ammonia-N	17·4	14
Amide-N	4·1	3·3
Amino-N	23·4	18·5
Other N	80·0	64·1
Solids	2180·0	—
Ash	286·0	—

Precipitation with Neuberg's reagent

The solution was mechanically stirred and 40 % Na_2CO_3 added until the liquid was acid to phenolphthalein but alkaline to litmus. After standing 2 hr., 21 g. of inorganic solids were removed by filtration. To the filtrate were added in small alternate quantities 1900 g. Na_2CO_3 (in 10 % solution) and 4750 g. mercuric acetate (in 25 % solution) until red mercuric oxide began to separate. After a day at 4–5° the voluminous precipitate was filtered off and washed with 80 % alcohol. The filtrate and washings were combined and will be referred to later as the 'Neuberg filtrate'.

The Neuberg precipitate was suspended in as small a quantity of water as possible and treated with H_2S. When the liberation of the nitrogenous material was complete, the solution was kept at 70° for a number of hours to granulate the mercuric sulphide. After filtering and washing with hot water, the filtrate and washings were concentrated *in vacuo* at 35° and the volume made up to 2 l. The analysis at this stage is given in Table 4.

On comparing Tables 3 and 4, it will be seen that the Neuberg precipitate has carried down 82 % of the total N of the original solution, and that the amide-N has disappeared, probably by hydrolysis in the alkaline medium, thus augmenting the yield of ammonia-N.

Table 4

	g. in 2 l. ml.	% of total N
Total N	101·9	100
Ammonia-N	19·0	18·65
Amide-N	None	None
Amino-N	23·9	23·5
Other N	59·0	57·9
Solids	1094·2	—
Ash	211·8	—

H_2SO_4 was next added to the above-mentioned solution obtained from the Neuberg precipitate until the concentration of the acid was 5%; this was followed by 25% aqueous phosphotungstic acid containing 5% H_2SO_4, added in small quantities at a time with stirring, until a drop of the supernatant liquid gave a white precipitate with a drop of baryta. The reaction mixture was kept at 5° for 50 hr., filtered and the precipitate washed well with 5% H_2SO_4. The procedure up to this point has separated the original solution of serum solids into three fractions (cf. Table 1):

(*a*) 'Neuberg filtrate': a fraction containing nitrogenous substances not precipitated by mercuric acetate-sodium carbonate.

(*b*) 'Phosphotungstic acid precipitate': a fraction containing nitrogenous substances precipitated by mercuric acetate-sodium carbonate and by phosphotungstic acid, viz. purines, diamino-acids.

(*c*) 'Phosphotungstic acid filtrate': a fraction containing nitrogenous bodies precipitated by mercuric acetate-sodium carbonate but not by phosphotungstic acid, viz. monoamino-acids.

Neuberg filtrate

The Neuberg precipitate having been removed, the filtrate was diluted with an equal volume of 95% alcohol (18 l.) and kept for 24 hr., after which some mercuric acetate was removed as a co-ordination compound with mercuric oxide [Berthelot, 1883]. The filtrate was now acidified with acetic acid and H_2S passed in to remove traces of mercury. After filtration the combined filtrate and washings were concentrated *in vacuo* at 35° to 8 l. Since 1900 g. of Na_2CO_3 had been introduced into this solution during the Neuberg precipitation and since the sodium was now present in the form of both acetate and carbonate, the following method was used to remove the sodium ion as chloride and the acetate ion as acetic acid.

The solution was first acidified to *p*H 3 with HCl. This caused the separation of a large quantity of NaCl and liberated all the acetic acid. The salt was removed by filtration and the filtrate concentrated until sufficient NaCl had separated to make the vacuum distillation difficult. The salt was again removed and distillation continued. In this way practically all the acetic acid was removed and the volume of the solution reduced to 2·5 l. At this point 2·5 l. of 95% alcohol were added to the concentrate, whereby 500 g. of NaCl and KCl were removed. The clear filtrate was reduced *in vacuo* to 1 l. and an equal volume of alcohol added. This threw down much quebrachitol and some inorganic material. These were filtered off and washed with alcohol. The filtrate and washings were concentrated to remove all alcohol, and then submitted to the continuous butyl alcohol extraction of Dakin [1918; 1920] for 200 hr. to remove any proline that might have escaped precipitation by the Neuberg reagent. It was found that the butyl alcohol extracted a fair amount of material, including most of the pigment, from the aqueous solution, and that on removing the solvent the

resulting thick paste was very soluble in water. 5 g. of this paste were treated for proline by the method of Dakin [1920]. To the solution in 50 ml. of water 8 g. of potassium cyanate were added and the whole evaporated to dryness on the water bath. The mass was taken up with water and acidified with H_2SO_4 until acid to Congo red (*p*H 3). The solution was then extracted with ether for 7 hr. in a continuous extractor [Dakin, 1920] to remove monoamino-acids, if present. When the extraction was complete 1/5 vol. of H_2SO_4 (50 %) was added and the solution heated on the water bath for 2 hr. to convert the proline into the hydantoin. The solution was again extracted with ether for 50 hr. when there were obtained 26 mg. of substance which melted at 141–144°. The melting-point of *i*-proline hydantoin is given by Dakin [1920] as 143°. (Found by Folin's colorimetric method [1925]: N, 21·1 %; $C_6H_8O_2N_2$ requires N, 20·0 %.) The small yield did not permit of further confirmatory evidence being obtained. Small-scale experiments using mercuric chloride gave no evidence for the presence of betaines in this fraction.

Phosphotungstic acid precipitate

The wet precipitate was suspended in 3 l. of water and decomposed by adding baryta until alkaline to litmus. The magma was kept warm for 3 hr., filtered, the barium phosphotungstate well washed with hot water and the filtrate and washing reduced *in vacuo* to 3 l. The last trace of barium was now removed in the usual way and the final volume adjusted to 2 l. This is the 'purine and diamino-acid fraction', which analysed as follows:

	g.
Total solids	203·6
Ash	98·4
Total N	18·6

The fraction was treated with $AgNO_3$ (120 g.), which threw down a very dense canary yellow precipitate of purine-silver nitrate complexes. This was filtered, washed with hot water and decomposed with H_2S. The filtrate on evaporation gave 91 g. of a mixture of yellow crystals and a dark-brown syrup having an ash content of 65·1 %, the residue being almost pure tungstic oxide. The mixture contained 2·8 g. N, but attempts to separate the purine bases from it were unsuccessful.

The histidine fraction. The above-mentioned filtrate, which might contain the three bases, was submitted to the usual Kossel procedure for their isolation. Baryta was added until a drop of supernatant liquid no longer gave a white precipitate with a reagent made by adding ammonia to a 10 % silver nitrate solution until the silver just dissolves. The precipitate was kept in the cold for several hours before filtering and washing with cold water. It was then decomposed with H_2S and the clarified filtrate taken to dryness *in vacuo*; 1·5 g. of a light lemon-yellow coloured solid were obtained, which gave none of the usual reactions for histidine. It is inferred, therefore, that the latex serum does not contain this base.

The arginine fraction. The base solution was next saturated with baryta at 40°. After several hours the arginine-silver complex was separated by filtration, decomposed in the usual way and concentrated under reduced pressure. The solid material thus obtained weighed 4 g., and the ninhydrin test showed that α-amino-N was present. Arginine was obtained from it as flavianate by the method of Vickery [1926] and for further characterization was converted into the carbonate by the method of Pratt [1926]. The yield of white crystalline

material was 1·42 g.: 0·2410 g. when heated at 105° for 4 hr. lost 0·104 g., showing the presence of 0·5 mol. of water of crystallization (no previous reference in the literature). For the air-dried material: C = 37·6; H = 6·66 %. $C_7H_{14}O_4N_4$, 0·5 H_2O requires C, 36·95; H, 6·61 %. For the anhydrous material: N = 25·15 %. $C_7H_{14}O_4N_4$ requires N, 25·7 %. The silver salt of arginine was made from 0·2 g. by adding sufficient HNO_3 to form the mononitrate and then adding the equivalent of silver nitrate. On diluting with alcohol, then with ether and keeping the mixture in the dark, white needles separated slowly (M.P. 181–183° with decomposition; Riesser [1906] gives 183°). Found: Ag, 26·64 %. $C_6H_{14}N_4O_2.HNO_3.AgNO_3$ requires Ag, 26·15 %. The picrate melted at 203–205° (Mulliken [1916] 205–206°) and the anhydrous copper salt at 238–240° (Riesser [1906] 232–234°). A solution made by dissolving 1 % of the carbonate in 5 % HCl showed in a 1 dm. tube $[\alpha]_D^{20} + 9·1°$.

From the above evidence it is certain that the compound isolated was *d*-arginine and that it therefore occurs as such in the original solution of serum solids.

The lysine fraction. Silver and barium were completely removed from the arginine filtrate and the clarified solution concentrated to 510 ml. The mercuric chloride-baryta treatment of Winterstein [1904] gave 5 g. of a dark-brown hygroscopic mass from which it was not found possible to isolate lysine as its picrate. Other attempts to obtain some evidence for the presence of this base also failed.

Phosphotungstic acid filtrate

The amino-acid fraction which was precipitated by Neuberg's reagent but not by phosphotungstic acid was submitted to continuous extraction with butyl alcohol to give:

(*a*) A fraction soluble in butyl alcohol.

(*b*) A monoamino-acid fraction insoluble in, but extracted by, butyl alcohol.

(*c*) A monoaminodicarboxylic acid fraction not extracted by butyl alcohol.

It must be stated that this extraction was by no means as clear cut as in the case of hydrolysed proteins for which it was primarily designed, but even so a sufficient separation of material was obtained to lead to the isolation of a number of amino-acids.

The extraction was continued for 200 hr., the butyl alcohol being changed every 24 hr. At the end of each day the butyl alcohol was filtered to remove fraction (*a*) (see above) and the residue washed with fresh alcohol. The filtrates were collected, the alcohol removed *in vacuo* and the residue taken up in water (fraction (*b*)). The aqueous solution of unextracted material was evaporated *in vacuo* to remove butyl alcohol and some acetic acid, and there was thus obtained a thick golden-yellow paste (fraction (*c*)).

Fraction (*a*). The solution (500 ml.) on standing deposited 4·4 g. of nearly colourless crystals which gave a strong Millon reaction indicating the presence of tyrosine. The material was suspended in 150 ml. of 95 % alcohol and boiled, water being added slowly until no more of the suspension went into solution. The residue was filtered off and washed with alcohol. The dried material (0·69 g.) was dissolved in hot water, treated with norit (0·1 g.), filtered and concentrated on the water bath to incipient crystallization. On cooling, characteristic crystals of tyrosine separated and were filtered off (0·41 g.). The mother liquor on further concentration yielded an additional 0·11 g. The Millon test was positive. (Found: C, 59·9; H, 6·0; N, 7·69 %. $C_9H_{11}O_3N$ requires C, 59·7; H, 6·1; N, 7·73 %.) On standing, the solution from which the crude tyrosine had separated deposited

thin lustrous crystals. These were filtered off and washed with cold alcohol (1·62 g.). Millon's test was negative, showing the absence of tyrosine. Their greasy feel, the fact that they were moistened by water only with difficulty, and especially their analysis, suggested that they were leucine (found: N, 10·68; amino-N, 10·94 %; mol. wt. (freezing point of water) 126·7; $C_6H_{13}O_2N$ requires N, 10·7; amino-N, 10·7 %; mol. wt. 131), but the specific rotation in water, $[\alpha]_D^{20}-13{\cdot}6°$, was too low and in 20 % HCl was too high $[\alpha]_D^{20}+27{\cdot}1°$, the recorded values in the literature being $-10{\cdot}42°$ and $+15{\cdot}53°$ respectively. Further investigation showed that the product was a mixture, and *l*-leucine was identified as its phenylhydantoin, M.P. 119–120°, and *d-iso*leucine as its benzoyl derivative, M.P. 116°.

The mother liquor from the above separation was concentrated to a syrup and kept at 0° for 24 hr. A mass of crystalline material was deposited which was filtered off and washed with ethyl acetate. The product (6·73 g.) was recrystallized from hot water to give ill-defined colourless platelets (2 g.) which were subsequently shown to be a mixture of *l*-leucine and *d-iso*leucine. The final mother liquor was taken to dryness (473 g.) dissolved in the minimum quantity of water and treated with much alcohol. On standing, another crop of crystalline material (2·6 g.) was obtained. This was filtered off, dissolved in water and precipitated by adding a large volume of alcohol. The product was *d*-valine. (Found: C, 50·4; H, 9·45; N, 11·88 %. $C_5H_{11}O_2N$ requires C, 51·3; H, 9·40; N, 11·96 %.) The specific rotation in water was $[\alpha]_D^{20}+6{\cdot}3°$ and in 20 % HCl $[\alpha]_D^{20}+28{\cdot}6°$, while the phenylcarbamido-acid melted at 154–153° and the phenylhydantoin at 129–130·5°, values that are in keeping with those recorded in the literature.

Fraction (*b*). This weighed 7·8 g. and contained 30 % of ash. 2 g. of the material were dissolved in 10 ml. of *N* NaOH and to the cooled solution an equivalent of phenylisocyanate was added. The mixture was shaken, with cooling, until the odour of isocyanate had disappeared. After filtration the phenylcarbamido-acids in the filtrate were precipitated by adding 10 ml. of *N* HCl. On recrystallization 1 g. of the phenylcarbamido derivative of *iso*leucine, M.P. 119–120°, was obtained. The hydantoin obtained by dehydration with HCl melted at 80°.

Fraction (*c*). After standing for about two months this fraction showed no signs of crystallization; it was therefore saturated with gaseous HCl, which precipitated much sodium and potassium chlorides. These were removed by filtration, and the filtrate set aside at 4–5° for 5 weeks, but no glutamic acid separated. A similar result was obtained in attempting to isolate glycine ethyl ester. The material was therefore collected, water removed, the residue covered with 4 vol. of 95 % alcohol and saturated with dry HCl to precipitate most of the inorganic material (150 g.) and to bring the remainder into solution (as ethyl ester). After filtration, the alcohol and water were then removed *in vacuo*, 4 vol. of absolute alcohol were added, and the mixture again saturated with gaseous HCl.

The esterified solution was evaporated almost to dryness under reduced pressure and the residue dissolved in the smallest quantity of water. The esters were liberated with NaOH and extracted with ether after saturation of the liberated ester solution with K_2CO_3. When the greater part of the dry ether was evaporated, 6 vol. of distilled water were added and the solution refluxed for 6 hr. to hydrolyse the esters to the free amino-acids and ethyl alcohol. Ordinary distillation removed most of the alcohol and evaporation under reduced pressure left a solid residue weighing 38 g. and also gave a turbid distillate from which were obtained 2 ml. of a pungent-smelling oil. This oil was set aside to be

investigated at some future date. The solid residue was dissolved in as little hot water as possible and decolorized with animal charcoal. To the clear filtrate 500 ml. of acetone were added, which caused the separation in two crops of 16 g. of material, M.P. 259–263°. Appropriate tests showed that this contained no glutamic acid, but that the ninhydrin reaction was positive. Negative Millon and positive xanthoproteic tests indicated the presence of phenylalanine. It was found, after a great deal of experimentation, that this material was not completely soluble in cold glacial acetic acid; 4 g. were accordingly suspended in cold glacial acetic acid and filtered rapidly, the residue being washed repeatedly with small quantities of the acid. In this way 1·04 g. of a pure white crystalline substance were obtained. Analysis of the substance, and of its insoluble copper salt, showed that it was *l*-aspartic acid. (Found: C, 36·4; H, 5·27; N, 10·58%. $C_4H_7O_4N$ requires C, 36·1; H, 5·26; N, 10·52%.) The specific rotation in 1% aqueous solution was $[\alpha]_D^{20} - 4{\cdot}1°$. The copper salt contained 23·0% Cu; $C_4H_5O_4NCu, 4{\cdot}5H_2O$ requires Cu 23·05%.

Summary

The following amino-acids are shown to occur in the serum of the latex of *Hevea brasiliensis*, all except the last having been actually isolated from it: tyrosine, *l*-leucine, *d-iso*leucine, *d*-valine, *d*-arginine, *l*-aspartic acid, *i*-proline, phenylalanine. A cursory examination of the amino-acid composition of the heat-coagulable protein of latex has been made. A tabular summary is given of the results obtained by various workers to date on the amino-acid composition of latex and latex proteins.

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67. A COLORIMETRIC METHOD FOR THE DETERMINATION OF CITRULLINE[1]

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In the course of experiments on the mechanism of urea formation, we came to feel the need of a convenient method for the determination of small quantities of citrulline. The carbamido-diacetyl reaction described by Fearon [1939] appeared to offer a prospect in that direction. Fearon himself had utilized it in an approximate determination of the citrulline content of trypsin-digested casein, but in general his observations were qualitative. We are indebted to him for permitting, and indeed encouraging, us to explore independently the possibilities of his test as the basis of a quantitative method.

The reaction consists in the development of a red (in the case of urea a yellow) colour, when the reacting substance is heated with diacetyl monoxime in acid solution and the product subsequently oxidized with potassium persulphate. It is not specific for citrulline, being given apparently by all compounds of the general formula $R.NH.CO.NH_2$ and by some of the formula $R_1.NH.CO.NH.R_2$. To Fearon's list of substances tested and found to react positively we are able to add the α-carbamido derivatives of propionic, *n*-butyric, *n*-valeric, *iso*butylacetic, δ-guanidinovaleric acid and δ-aminovaleric acids, β-carbamidopropionic acid, ε-carbamidohexoic acid and 3-carbamido-2-piperidone [Hunter, 1938]. Among known tissue constituents, other than citrulline itself, the only ones that give the test are urea, allantoin, proteins and such higher protein derivatives as peptone and gelatin.

In adapting the reaction to the quantitative determination of citrulline we have employed the method of photoelectric colorimetry, using the instrument of Evelyn [1936]. This possesses the advantage over most similar forms of apparatus (of particular importance for the present application) that the reaction is carried out, and the colour developed, in the same special test tubes, which serve as the absorption cells of the colorimeter. The procedure recommended could doubtless be adapted to any other photoelectric colorimeter. For visual colorimetry it is hardly suitable.

Lacking facilities for a precise spectrophotometric study of the coloured reaction mixture, we had to be guided, in the choice of a suitable light filter, by an approximate procedure, in which the photocolorimeter itself is used as a spectrophotometer. A spectrogram of the red solution (for the preparation of which we are indebted to Dr Ireton and Dr Allin of the Department of Physics, University of Toronto) revealed an absorption band in the green and blue, extending roughly from 460 to 520 mμ. Following this indication, we applied the analytical procedure, presently to be described, to six 7 ml. samples of a 0·001 % citrulline solution, using with successive samples each in turn of the

[1] The method was described, and a preliminary account was given of some results already obtained with it, at a meeting of the Royal Society of Canada held at Kingston, Ontario, on 21 May 1941.

six Rubicon filters numbered (in correspondence with the dominant wave-length of the transmitted light) from 420 to 565 mμ. The six values for photometric density thus obtained were plotted against the filter numbers or dominant wave-lengths. The resulting curve suggested that the maximum of absorption occurs at or near 475 mμ. A filter ideal for that wave-length not being immediately procurable, No. 490 (transmission limits 465 to 530 mμ) was adopted as the best available compromise.

It may here be mentioned that we constructed similar rough absorption curves for urea, allantoin, methyl- and phenyl-urea, α- and β-carbamidopropionic acids, ε-carbamidohexoic acid and 3-carbamido-2-piperidone. For the four last of these the curves were to all appearance identical, both in form and position, with the curve for citrulline. From that pattern methyl- and phenyl-urea showed only minor deviations. The curve for urea, as was to be expected, was conspicuously different, its peak falling in the neighbourhood of 450 mμ. Allantoin yielded a curve differing little from that of urea.

In order to obtain consistent results in the quantitative application of Fearon's test it was found necessary to define very rigidly both the proportions of the reagents and the conditions under which the colour is developed. The technique we are about to describe, adopted after many exploratory experiments, not only ensures the reproducibility of the results, but enhances considerably the sensitivity of the reaction. It permits the estimation of citrulline in any concentration down to (for pure solutions) 0·2 or 0·3 mg./100 ml., with an error which, except at the very lowest concentrations, does not exceed 2% (see Table 2).

Description of the method

The following description takes for granted an acquaintance with the theory and the general operational technique of the Evelyn photoelectric colorimeter [Evelyn, 1936; Dann & Evelyn, 1938] and omits mention of details common to all methods in which that instrument is employed.

A constant-level water bath is provided with a cover and a perforated false bottom. The cover is pierced with a number of round holes, of a diameter (about 22 mm.) exactly fitting the absorption tubes of the colorimeter. The false bottom is fixed at such a level that the vertical distance between it and the cover is between 70 and 90 mm. The water level is adjusted so that the height of water above the false bottom is between 45 and 50 mm. The dimensions specified are such that during the period of heating only that part of the tube will be immersed which is charged with the reacting mixture, while half at least of its length will project above the cover. The objects sought are the standardization of the heating and the avoidance, as far as possible, of concentration through evaporation. The second of these objects requires that the projecting part of the tube should be kept as cool as possible. All the holes in the cover should therefore either be occupied by tubes or covered by small watch-glasses. It is of advantage also to have a number of holes in the wall of the bath below the cover, so that escaping steam will be directed away from the tubes.

While the water in the bath is kept gently, but steadily, boiling, a measured volume of the citrulline-containing solution is transferred to one of the special absorption test-tubes of the colorimeter. The volume should be such as to contain about 0·05–0·07 mg. of citrulline—in no case less than 0·02 or more than 0·12 mg. Water is added to make a total volume of exactly 7 ml. Alternatively the solution may first be diluted to a concentration of 0·7–1 mg./100 ml., 7 ml. being then measured into the absorption tube. If the concentration of citrulline is

quite unknown to begin with, one or more random trials will usually indicate the quantity or the dilution proper for a precise determination.

To the citrulline solution there are added (1) 4 ml. of conc. HCl (sp.gr. 1·18–1·19) and (2) 0·5 ml. of a 3 % solution of diacetyl monoxime. The reactants having been mixed, the tube is placed in the bath, and a 1 in. funnel is set in its mouth to act as a condenser. The tube is left in the boiling water for *exactly* 9 min. It is then removed, wiped dry and allowed to cool in the air for 6 min. This will reduce its temperature to about 65°. If citrulline is present, the mixture will already show a pink colour. To develop this more fully, there is now added one drop of 1 % potassium persulphate solution. The tube is thereupon placed in position in the colorimeter (already equipped with the appropriate filter, No. 490, and adjusted to the reagent-blank rest point), and the behaviour of the galvanometer spot is observed. As the colour deepens, the galvanometer reading, of course, falls. When it shows signs of becoming stationary, another drop of persulphate is added. If this produces a further fall, a third drop may be needed to make sure that the maximum attainable depth of colour and the minimum galvanometer reading have been reached. This is usually accomplished in 1–2 min. After a time the colour begins to fade, and the galvanometer reading to rise again. The lowest reading observed is the significant one. It is translated into terms of citrulline concentration by reference to a previously constructed calibration curve.

A calibration curve is necessary because, with the technique described, the logarithmic law of Lambert and Beer holds good only over a limited range of concentrations. The reason may be that the filter used is insufficiently selective, or that with the lower concentrations fading of the colour overtakes its full development. A curve for use with pure solutions may be constructed from the data recorded in Table 1. In practice only that part of the curve would ordinarily

Table 1. *Calibration curve for pure solutions of citrulline*

ml. of stock citrulline solution (5 mg./100 ml.) in a total volume of 7 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading (average of two to three trials)
0	0	100 (R.P. = 66)
0·3	0·214	88·75
0·6	0·429	73·7
0·9	0·643	59·9
1·2	0·857	48·0
1·5	1·071	38·0
1·8	1·285	29·4
2·1	1·50	23·7
2·4	1·715	19·3
2·7	1·93	16·25
3·0	2·14	13·3
3·6	2·57	10·0

be used, which lies between galvanometer readings of 80 and 20 (the respective citrulline concentrations being 0·35 and 1·7 mg./100 ml.). The concentration read from the curve is of course that existing in the volume of 7 ml. directly analysed. From this the concentration in the original solution is readily calculated.

The time occupied by a single determination (from the addition of the reagents to the final reading of the galvanometer) is 16–17 min., and so simple are the manipulations required, that a series of determinations can be carried along in orderly progression at regular intervals of 3 min.

To the foregoing description of the method we may add here the following comments. With a given amount of citrulline the depth of colour obtained depends upon the proportions of HCl and diacetyl, upon the intensity and duration of the heating and, in a smaller measure, upon the length of the subsequent cooling period. In the procedure prescribed the reagents are applied in what have been found to be the optimal concentrations with respect to colour development. The period of hearing, on the other hand, is a compromise. If it is shortened, less colour is produced, and the sensitivity of the method is correspondingly diminished. If it is lengthened, colour production and sensitivity are increased; but the method then becomes rather tedious. A change in either direction, while necessitating the construction of another calibration curve, is not only permissible, but might in special circumstances be of advantage. The essential point is not that the reaction mixture be heated for 9 min., but that both the time and the manner of heating be always exactly the same. The prescribed time of cooling need not be quite so rigidly adhered to.

Accuracy of the method with pure solutions

In the preparation of the calibration curve the agreement between duplicate or triplicate runs on any one concentration of citrulline was always within 2%. It was to be expected that in the analysis of pure solutions the results obtained would be equally good. This expectation is confirmed by the examples given in Table 2. Only occasionally does the error exceed the proportion named.

Table 2. *Analysis of pure solutions of citrulline of known concentration*

Concentration, mg./100 ml.	
Known	Found
0·236	0·22
0·59	0·58
0·67	0·67
0·75	0·73
0·75	0·75
0·826	0·83
1·00	1·00
1·00	0·99
1·00	1·02
1·46	1·46

Behaviour of reacting substances other than citrulline

In any practical application of the method, account has to be taken of certain difficulties, the first of which is the lack of specificity of the carbamido-diacetyl reaction. Among the substances which give this reaction there are at least three which may be expected to be present in practically every animal tissue or fluid: protein, urea and (except in human material) allantoin.

Protein and its higher derivatives occupy an exceptional position in that they yield, under the conditions prescribed, not only what appears to be a citrulline reaction, but also an additional violet colour (Liebermann reaction) partially or completely obscuring the other. Fearon discusses means of minimizing this effect in the qualitative test, but we have found no satisfactory way of preventing or compensating for it in the quantitative technique. Any solution to be analysed for free citrulline must therefore first be rendered protein-free. Among possible deproteinizing agents the most suitable is trichloroacetic acid. Uranyl acetate and tungstic acid may also be employed, although with the

latter care must be taken to avoid such an excess, as would precipitate on the addition of the hydrochloric acid.

In order to evaluate the disturbing effects of urea and allantoin we analysed solutions of each in routine fashion, just as if they were solutions of citrulline. As a matter of interest (although of less immediate practical importance), we also treated in the same way solutions of the other carbamido compounds listed in Table 3. Each substance was tested in at least two concentrations. These are stated in the third column of the table, while in the fourth there is shown for each that concentration of citrulline to which, in carbamido content, it is equivalent. The fifth column records the citrulline concentrations which the method appeared to indicate, while in the last the 'citrulline' thus found is expressed as a percentage of the citrulline equivalent. For citrulline itself the figure in the last column is of course 100.

Table 3. *Behaviour of various substituted ureas in the proposed method for citrulline*

No.	Substance	Concentration tested mg./100 ml.	Equivalent concentration of citrulline mg./100 ml.	Apparent citrulline concentration found	
				mg./100 ml.	% of citrulline equivalent
1	Urea	0·4	1·17	0·43	36·7
		2·0	5·84	1·56	26·7
		4·0	11·67	1·86	15·9
2	Allantoin	10·0	11·1	0·86	7·7
		20·0	22·2	1·33	6·0
3	Methylurea	0·212	0·50	0·55	110·0
		0·846	2·00	2·00	100·0
4	Phenylurea	0·39	0·50	0·38	76·0
		0·777	1·00	0·75	75·0
5	α-Carbamidopropionic acid	10·0	13·3	0·46	3·5
		20·0	26·5	1·04	3·9
6	α-Carbamido-*n*-butyric acid	10·0	12·0	0·24	2·0
		20·0	24·0	0·51	2·1
7	α-Carbamido-*n*-valeric acid	10·0	10·9	0·24	2·2
		20·0	21·9	0·57	2·6
8	α-Carbamido*iso*hexoic acid	10·0	10·0	0·17	1·7
		20·0	20·0	0·42	2·1
9	α-Carbamido-δ-guanidino-*n*-valeric acid	10·0	8·1	0·19	2·3
		20·0	16·1	0·38	2·4
10	α-Carbamido-δ-amino-*n*-valeric acid	10·0	10·0	0·18	1·8
		20·0	20·0	0·38	1·9
11	β-Carbamidopropionic acid	0·377	0·50	0·375	75·0
		0·754	1·00	0·775	77·5
12	δ-Carbamido-α-amino-*n*-valeric acid (citrulline)	—	—	—	100·0
13	ε-Carbamido-*n*-hexoic acid	0·50	0·50	0·56	112·0
		1·00	1·00	1·11	111·0
14	3-Carbamido-2-piperidone	10·0	11·2	0·75	6·7
		20·0	22·3	1·56	7·0

The reactivity of allantoin is seen at once to be of a very low order. It seems unlikely that in conventionally prepared blood or tissue filtrates one would ever encounter an allantoin concentration as great as 1 mg./100 ml. Table 3 shows that this would be equivalent to 1·11 mg. of citrulline, but would yield, over the spectral wave-band employed, only 7·7 % as much colour—would simulate, that is to say, a citrulline concentration of only 0·086 mg./100 ml. In the uses to

which we have hitherto put the method, this would be a negligible quantity. At the same time it must be recognized that substantial concentrations of allantoin, such for example as might occur in urine, would seriously complicate the application of the method.

The possible influence of urea is more important. A 1:10 protein-free filtrate of normal blood would contain about 3 mg./100 ml. of urea. From the data in Table 3 it may be estimated, by interpolation, that this would give about 20% as much colour as the equivalent amount (8·76 mg.) of citrulline. In the complete absence, therefore, of citrulline itself, the filtrate would show an apparent citrulline content of as much as 1·75 mg./100 ml. Similarly it may be calculated that a saline tissue extract containing, when diluted for a citrulline determination, 1 mg./100 ml. each of citrulline and urea (a combination such as has frequently arisen in our experiments) would yield, by the prescribed method, a citrulline value (1·95) almost double the true one. Since the urea colour is different from the citrulline one, the difficulty could perhaps be met by making two measurements of photometric density, each with a different, appropriate and sufficiently selective filter; but it is simpler to destroy the urea with urease, and this expedient we have accordingly adopted as a necessary preliminary to the determination of citrulline in any biological fluid. The destruction of urea can of course be conducted as a step in its manometric determination [Krebs & Henseleit, 1932]. Afterwards the urease can be removed, along with pre-existing proteins, by any suitable reagent.

Urea and allantoin are the only substances in Table 3 which come under consideration as regular tissue constituents. The others (citrulline itself excepted) are of interest only from a theoretical point of view. Six of them (Nos. 3, 4, 5, 11, 13 and 14) have been shown to yield, in the diacetyl reaction, products with absorption spectra differing little, if at all, from that of the citrulline product. As far as visual observation goes, the colour given by the remaining five (Nos. 6–10) is of exactly the same reddish shade. For all of these therefore (but not for urea or allantoin) the figures in the last column of Table 3 indicate the relative intensity with which the carbamido group reacts in the various combinations represented.

Regarded from this point of view the data show that, as a test for substituted ureas, the carbamido-diacetyl reaction varies in sensitivity with the character of the substituting group. Thus methylurea gives about the same depth of colour as an equivalent quantity of citrulline, but phenylurea gives only three-fourths as much, and piperidonylurea only one-fifteenth. Of particular interest are the results within the special group of the carbamido-acids, to which citrulline itself belongs. Here the intensity of the reaction appears to depend directly upon the distance separating the urea residue from the carboxyl. With α-carbamido-acids the reaction is feeble, and is little, if at all, affected by either the length of the fatty acid chain or the presence of such other substituents as the amino- or guanidino-group. The β-acid gives about 25 times more colour than the average α-acid, but not so much as the δ-acid, citrulline. The reactivity of the latter is surpassed, in turn, by the ε-acid. As one special example of these relations, citrulline is seen to react more than 50 times as intensely as its isomer, α-carbamido-δ-amino-*n*-valeric acid (No. 10).

Application of the method in tissue analysis

The determination of citrulline in biological material (in blood, for example, or in tissue extracts) requires, as has been shown, the prior removal of urea and proteins; but, even with this accomplished, other complicating factors have still

to be dealt with. In the first place most urea- and protein-free filtrates, devoid though they may be of citrulline, still contain a trace of material giving the carbamido reaction. This trace may be too small to give a visible pink colour, yet large enough to simulate in the photocolorimeter a not entirely negligible content of citrulline. If this were all, the effect might be allowed for by deducting from all results with any particular material a constant 'blank', determined on similar material known to contain no citrulline. A more serious complication is, that tissue filtrates contain also something, which modifies in a peculiar way the development of the colour from any citrulline actually present. The effect is, that with low concentrations of citrulline colour development is greater, with high concentrations less, than in pure solutions. Under these circumstances the calibration curve already described might still give reasonably correct results for certain medium concentrations, but for all others it would be seriously misleading. It becomes necessary therefore to construct, for each practical application of the method, a special calibration curve.

The general procedure to be followed may be exemplified by describing in detail that application with which we have been most immediately concerned—the determination of citrulline, along with urea, in a saline medium containing the products derived from the metabolism of tissue slices. For the urea determination, to be carried out according to Krebs & Henseleit [1932], 3 ml. of the medium, it will be assumed, have been transferred to a suitable manometer vessel, and have then been treated with 0·3 ml. of acetate buffer (*p*H 5·0) and 0·3 ml. of urease solution. The manometer readings having been taken, the whole mixture is washed into a 15 ml. flask to a volume of about 12 ml. To this there is added 1 ml. of 50% trichloroacetic acid, whereupon the flask is heated for 2–3 min. in boiling water. After having been cooled, the flask is filled to the mark with water, its contents are mixed and it is kept for half an hour. The contents are then filtered, and of the filtrate 7 ml. (or some suitable smaller volume diluted to 7 ml.) are taken for analysis.

The analysis is carried out in the manner already described, except that galvanometer readings are referred to a special calibration curve. In the case under consideration the preparation of this special curve is simplified by the fact that the blank, and also the materials which disturb the development of the colour, are derived exclusively from the added urease. The data for the curve, which are given in Table 4, were accordingly obtained as follows. Into a series of 15 ml. volumetric flasks were measured the stated volumes of a solution containing in 100 ml. 14·6 mg. of citrulline. To each were added 0·3 ml. of acetate buffer and 0·3 ml. of the urease solution employed in the determination. The mixtures were then diluted to about 12 ml., deproteinized, brought to volume

Table 4. *Calibration curve for urease filtrates*

ml. of stock citrulline solution (14·6 mg./100 ml.) in a total volume of 15 ml.	Final citrulline concentration (mg./100 ml.)	Galvanometer reading
0	0	100 (R.P. = 75)
0·25	0·243	73·5
0·50	0·487	60·5
0·75	0·73	49·5
1·00	0·973	40·5
1·25	1·216	33
1·50	1·460	26·5
1·75	1·703	21·75
2·00	1·946	18

and filtered as already described. From the first filtrate, containing no citrulline and serving therefore as the blank control, 7 ml. were transferred to an absorption test tube, and the colour was developed in the regular way. When the photometric density had, upon careful addition of the oxidizing reagent, reached its maximum, the galvanometer was quickly set to 100. The tube was thereupon removed, and the 'rest point' noted. The colour was then developed in the remaining tubes, galvanometer readings being taken with adjustment each time to the rest point already determined. In this way the blank was automatically allowed for in each of the readings, while the effect of other interfering factors was reflected in the course of the curve relating the galvanometer readings to the known concentrations of citrulline.

The curve given by the data of Table 4 starts at a higher level than that drawn from Table 1, but, falling more steeply, crosses it at a citrulline concentration of about 1 mg./100 ml. It is applicable, but applicable only, to all analyses carried out on 7 ml. samples of filtrates prepared, in the way prescribed, from similar material treated with a urease solution of identical origin and concentration. An ideal requirement is that the rest-point obtaining during any series of determinations should be identical with that found during the preparation of the calibration curve. In practice a difference of several points is admissible.

For any other special application of the method another calibration curve, constructed on the same principles, would have to be prepared.

With respect to the urease it is of advantage, naturally, to employ a preparation which, in the quantity requisite, gives the lowest blank and the least interference. A crude jack-bean extract is, from this point of view, rather unsuitable. We have found it best to use a urease powder prepared from jack-bean meal by the method of Van Slyke & Cullen [1914] but further purified by a second extraction and a second precipitation with acetone. A 10% suspension of this powder is of ample potency, and yields (with 7 ml. of filtrate) a blank of only 0·2 mg./100 ml. It was such a preparation that was used in securing the data of Table 4.

To test the accuracy of the whole procedure we used it, exactly as described, in the analysis of known mixtures of urea and citrulline. The results, as shown in Table 5, were entirely satisfactory.

Table 5. *Analysis of citrulline-urea mixtures*

Mixtures analysed					Urea concentration (mg./100 ml.)		Citrulline concentration (mg./100 ml.)	
ml. of urea solution 27·3 mg./100 ml.	ml. of citrulline solution 16·5 mg./100 ml.	Water to ml.	ml. acetate buffer (*p*H 5·0)	ml. urease 10% solution	Present	Found	Present	Found
0·5	0·3	3	0·3	0·3	4·55	4·47	1·65	1·60
1·0	0·6	3	0·3	0·3	9·10	8·97	3·30	3·35
1·5	1·2	3	0·3	0·3	13·65	13·23	6·60	6·70
2·0	0·9	3	0·3	0·3	18·20	17·65	4·95	4·85

In conclusion it may be reported that the method has enabled us to demonstrate the production by liver slices of substantial quantities of citrulline from ornithine, and thus for the first time to establish citrulline as an actual product of tissue metabolism. A full account of the relevant observations is in process of preparation.

SUMMARY

The carbamido-diacetyl reaction of Fearon is made the basis of a method whereby, through the use of a photoelectric colorimeter, small quantities of citrulline may be estimated with an error not exceeding 2%. Procedures are described for overcoming certain difficulties that arise in the application of the method to tissue analysis. Incidentally a comparison is made of the reactivities to Fearon's test of various carbamido-acids and other urea derivatives.

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68. HEMICELLULOSE PREPARATION AND CHLORINATION CELLULOSE

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It has recently been shown that the use of boiling alcoholic-NaOH pretreatment of wood as a preliminary to hemicellulose extraction results in a marked destruction of furfuraldehyde-yielding materials [Preece, 1940]; the extent of the destruction is such as to make the use of this pretreatment undesirable, even though it may reduce the lignin contamination of subsequent hemicellulose preparations. At the same time, some polyuronide material may pass into the alcoholic extract [see, e.g., Preece, 1931]. The nature of this material has hitherto been obscure, though there has been little reason to suppose that it differed greatly from hemicelluloses prepared by more normal means of extraction. The preparation of the so-called 'lignosaccharide' has now been studied in some detail and, as will appear, it is clear that it does not represent a special type of cell-wall constituent.

Hemicellulose nomenclature has been extremely confused, and it seems that any mention of these substances should be accompanied by a precise definition of what is intended. The position has been reviewed by Norman [1937], who emphasizes that material passing into hemicellulose preparations has a double origin. There is reason to suppose that the 'crude cellulose' remaining after alternate chlorination and sulphite treatment of plant materials represents the essential structural material of the tissues present; it is regarded as comprising 'pure' cellulose with associated cellulosan, the latter in hard-woods appearing to consist very largely of xylan. A variable proportion of this xylan is soluble in dilute NaOH, as is also a proportion of glucosan material. Whether this readily soluble glucosan should be regarded as cellulosan is not clear. Again, the crude cellulose is associated with encrusting materials which are removed by chlorination. These encrusting materials also are soluble to a greater or less degree in dilute alkali and so contribute pentosan, uronic anhydride and possibly in some cases hexosan to the hemicellulose. If this view is correct, therefore, the mixed nature of hemicellulose preparations can hardly be contested, and further evidence that the preparations are mixtures is presented below. It might be suggested that the term hemicellulose should be reserved for the encrusting urono-polysaccharides, the cellulosans being regarded merely as part of the true native cellulose. However, it is perhaps too soon to endeavour to define the various types rigidly and, in general, Norman's tentative nomenclature will be followed, hemicellulose implying urono-polysaccharide material soluble in dilute alkali, irrespective of its origin, though excluding pectin and materials capable of extraction with water.

Experimental

Of the two wood samples used, the mahogany sawdust was from a commercial timber. The willow material used was from two 5-6-year-old branches cut from a growing tree, the tissues down to the wood being removed and dis-

carded before preparation of the sawdust; in this case, a period of some 3 months elapsed between the cutting from the tree and the preparation of the analytical material.

Alcoholic-NaOH extraction

The mahogany material was extracted successively with ether, 95 % ethyl alcohol, 0·5 % ammonium oxalate solution and 95 % alcohol, each extraction being carried out at boiling temperature for 1 hr. and being followed by appropriate washings on a cloth filter. The loss of dry matter was 8·96 %. A modified and probably more efficient pretreatment of the willow sawdust involved successive extractions with 95 % alcohol, 0·5 % ammonium oxalate solution (twice), distilled water and 95 % alcohol (twice), each extraction being for 1 hr. as before; the dry weight loss was 5·77 %. It may be noted that the willow material used all passed a 20-mesh sieve; the mahogany was used as received. The following treatments and determinations were carried out on the air-dried pre-extracted materials:

(*a*) Furfuraldehyde was determined directly on 0·5–0·7 g. of the prepared wood, the distillation being made in presence of 25 ml. each of *N* NaOH and *N* HCl.

(*b*) A similar weight of the wood was extracted with 100 ml. of boiling x % ethyl alcohol-1 % NaOH for 1 hr. The NaOH was neutralized by adding 25 ml. *N* HCl, and the alcohol was removed *in vacuo*. Furfuraldehyde determination was carried out after the necessary adjustment of volume. The values of x used were successively 10, 30, 50, 70 and 90 %.

(*c*) Extractions were carried out as in (*b*), the mixture then being filtered at the pump and the wood residue washed (using small portions of liquor for each wash) with (i) 50 ml. of liquid having the same composition as that used for extraction, and (ii) 75 ml. of aq. alcohol of the same concentration as that already used. The residue was transferred to the distillation flask and 25 ml. each of *N* NaOH and *N* HCl were added, furfuraldehyde distillation then being proceeded with.

(*d*) Owing to absorption of alkali by the wood, the 25 ml. of *N* HCl added in (*b*) did not give a neutral mixture. Since the figures obtained for 'extract' (*b*) − (*c*) were somewhat anomalous with the 70 and 90 % alcoholic treatments, it was suspected that this might be due to a loss of furfuraldehyde during the vacuum distillation, as a result of the somewhat acid conditions prevailing. Accordingly, samples of wood were refluxed with 100 ml. of alcoholic-NaOH as in (*b*), and the cooled mixture was titrated with *N* HCl by the conductometric method. Assuming that the titre required was y ml., addition of HCl was continued to 25 ml.; $(25-y)$ ml. of *N* NaOH were then added, the further treatment being as in (*b*). This treatment was applied only to willow. The end-point was by no means sharp, though a good approximation was probably obtained. The absorption of alkali corresponded to 2·0–2·2 ml. of *N* NaOH per g. of wood.

The results of the treatments, calculated as furfuraldehyde % of the original, dry, unextracted woods, are shown in Table 1. The standard deviation of the mean of quadruplicates is ± 1·06 % of that mean for mahogany, and ± 0·61 % of that mean for willow; the greater precision in the latter case is due to the greater homogeneity of the sample. The standard deviations of differences, (*a*) − (*b*) and (*b*) − (*c*), are calculated independently in each case from the standard deviations of the pair of quadruplicate means concerned.

It will be observed that the results of treatment (*d*) do not differ significantly from those of (*b*), though it may be more than coincidence that degradation

Table 1. *Furfuraldehyde as percentage of original wood*

	Control (*a*)	Residue + extract (*b*)	Residue + extract (*d*)	Residue (*c*)	Degradation loss (*a*) − (*b*)	Extract (*b*) − (*c*)
Mahogany	9·17	—	—	—	—	—
1 % NaOH, alcohol 10 %	—	8·53	—	7·62	0·64 ± 0·132	0·91 ± 0·121
30 %	—	8·70	—	7·99	0·47 ± 0·134	0·71 ± 0·125
50 %	—	8·70	—	8·42	0·47 ± 0·134	0·28 ± 0·128
70 %	—	8·60	—	8·61	0·57 ± 0·133	—
90 %	—	8·56	—	8·74	0·61 ± 0·133	—
Willow	14·55	—	—	—	—	—
1 % NaOH, alcohol 10 %	—	13·00	13·08	10·38	1·55 ± 0·119	2·62 ± 0·101
30 %	—	13·14	13·20	11·32	1·41 ± 0·120	1·82 ± 0·106
50 %	—	13·24	13·26	12·28	1·31 ± 0·120	0·92 ± 0·110
70 %	—	13·12	13·14	13·39	1·43 ± 0·120	—
90 %	—	13·04	13·19	13·08	1·51 ± 0·120	—

with (*d*) is represented by smaller values throughout than with (*b*). However, (*d*) need not be discussed further.

The similarity of behaviour of the two woods is very great. With both the degradations losses show little change with variations in alcohol concentration, though the loss with willow is relatively and absolutely greater than with mahogany. The extract behaviours are likewise similar, though willow yields a considerably greater amount of material soluble in 10, 30 and 50 % alcoholic-1 % NaOH. There is a slight irregularity in the results from the 70 and 90 % extracts, but the figures obtained for the difference (*b*) − (*c*) are not significant (with the possible exception of the 70 % willow extract) and this matter may at present be ignored. The extracts containing up to 50 % of alcohol have removed the equivalents of amounts of furfuraldehyde which diminish as the alcohol concentration increases, and it is apparent that the extraction of furfuraldehyde-yielding material has substantially stopped by the time the alcohol concentration reaches 70 %. The regular decrease in extraction appears to support the contention that we are not dealing here with any special type of hemicellulose.

Working with straws, Norman [1935] reported an apparently greater extraction of polyuronides with 30 % alcohol-1 % NaOH than with aq. 1 % NaOH. With willow, aq. 1 % NaOH gave an extract furfuraldehyde of 2·40 % with a degradation loss of 2·16 %. The degradation loss is significantly greater than in any of the alcohol treatments, and it is obviously extremely difficult to attempt comparison between the present results and those of Norman, which took no account of the destruction of furfuraldehyde. Comparison is rendered further inadvisable by the fact that crude straw was used, whilst the willow had undergone preliminary treatment which included extraction with alcohol. Balancing any repressive effect that alcohol may have on degradation, it has previously been shown [Preece, 1940] that material soluble in alcohol may protect furfuraldehyde-yielding material against destruction; it may well be that such alcohol-soluble material also impedes extraction of hemicelluloses, dissolution being then favoured by even low concentrations of alcohol in the extracting medium.

Qualitative extraction

The following general method of 'lignosaccharide' and hemicellulose preparation was applied to fresh samples of both sawdusts, 750 ml. of each extractant being used per 100 g. of wood. Extraction was carried out at boiling temperature for 1 hr. each with: (i), (ii) aq. ammonium oxalate solution; (iii) distilled water; (iv), (v) *x* % alcohol-1 % NaOH; (vi), (vii) *x* % alcohol. The wood

residue was dried in air and reserved; it is designated lr_x. The values of x used were 10, 30 and 50 %. Extracts (i), (ii), (iii) and (vii) were discarded, but (iv), (v) and (vi) were combined and filtered through pulp. The volume of N HCl necessary to adjust the whole to neutrality was determined by titration of an aliquot portion by the minimum conductance method. The neutralized extract was concentrated under reduced pressure to about 1/4 vol., further concentration not being attempted since there is reason to suppose that high salt concentrations may interfere with hemicellulose precipitation. An equal volume of acetone was added and the precipitate, l_x, was purified as described later. Precisely one-half of the residue lr_x was extracted for successive 2-hr. periods with (viii), (ix) and (x) aq. 4 % NaOH at room temperature; repeated washings with water were then applied, after which the residue—hr_x—was dried in air and reserved. Hemicelluloses—h_x—were precipitated from the combined and filtered NaOH extracts by acidifying with HCl and adding an equal volume of acetone.

A further hemicellulose preparation, h, was made by extracting the wood by methods (i), (ii) and (iii), the extracts being discarded. The residue was then treated as under (viii), (ix) and (x), the hemicellulose being precipitated from these three combined extracts. The washed and dried wood residue is designated hr.

A measure of purification of the mahogany preparations was obtained by dissolving or suspending them in 2 % NaOH and reprecipitating with acetone after acidifying with HCl. The willow preparations, on the other hand, were all passed through the Cu purification method, after filtration of their NaOH solutions. Analyses of the products are shown in Table 2.

Table 2. *Analysis of hemicelluloses*

Preparation		Yield, on dry wood %	On dry, ash-free hemicellulose: Lignin %	On dry, ash-free hemicellulose: Furfuraldehyde %	Furfuraldehyde on dry, ash-free, lignin-free material %
Mahogany	h	2·30	4·33	54·26	56·72
	h_{10}	1·90	1·67	53·33	54·24
	h_{30}	2·11	2·23	55·81	57·08
	h_{50}	2·15	1·45	55·21	56·02
	l_{10}	1·72	21·34	31·48	40·02
	l_{30}	0·70	25·47	27·59	37·02
	l_{50}	0·16	35·38	21·90	33·89
Willow	h	10·64	1·06	57·53	58·15
	h_{10}	5·61	0·79	57·34	57·79
	h_{30}	7·00	0·98	57·82	58·39
	h_{50}	8·92	0·85	56·26	56·74
	l_{10}	3·07	7·22	48·12	51·92
	l_{30}	1·38	6·03	49·41	52·58
	l_{50}	0·22	11·90	37·63	42·71

Much of the lignin of the mahogany preparations of the l_x series has been carried down mechanically; thus, mahogany l_{10} was purified from a filtered NaOH solution by Cu treatment and then gave (on the ash-free basis): furfuraldehyde 38·09 %, lignin 5·13 %. Correcting the furfuraldehyde result for the lignin content the figure becomes 40·15 %; evidently the purification has not modified the composition of the lignin-free material, of which 82 % was recovered by the technique adopted. It is clear that alcohol and acetone washes alone cannot be relied on for removal of lignin. The alcoholic-NaOH pretreatment has markedly reduced the lignin content of mahogany preparations h_x as compared

with h; with willow the corresponding differences are insignificant. It appears that, with both woods, the Cu-purified preparations l_x give markedly more lignin than the corresponding members of the h_x series. The variations in furfuraldehyde yield for h and h_x from a given wood are small and somewhat irregular; these furfuraldehyde yields always exceed those for l_x, even after correction for lignin. The yields of l_x show again the restricting effect of increasing alcohol concentration.

The wood residues

The seven residues from each wood, and the original woods themselves, were examined for furfuraldehyde yield and for their content of Cross and Bevan cellulose, the furfuraldehyde yield from this crude cellulose being also determined. No pretreatment was applied in the cellulose determination, except that the original wood samples were extracted with alcohol-benzene mixture. Chlorination was for periods of 20 and 15 min., followed by further treatments as required of 10 min. each. The results are shown in Table 3, where the compositions of the crude celluloses are also given, it being assumed that the furfuraldehyde is derived exclusively from xylan, pure cellulose (glucosan) being obtained by difference.

Table 3. *Analysis of wood residues*

	Wood	lr_{10}	lr_{30}	lr_{50}	hr_{10}	hr_{30}	hr_{50}	hr
Mahogany:								
On dry wood or residue, %:								
Total furfuraldehyde	9·30	9·88	9·95	10·01	8·22	8·19	8·38	8·20
Cross and Bevan furfuraldehyde	5·02	6·84	6·86	6·71	6·23	6·37	5·68	5·92
Chlorination loss	4·28	3·04	3·09	3·30	1·99	1·82	2·70	2·28
Cross and Bevan cellulose	53·64	63·92	62·63	61·34	65·30	67·75	65·01	62·55
On Cross and Bevan cellulose, %:								
Glucosan	85·5	83·4	83·0	83·0	85·2	85·4	86·4	85·3
Para-xylan	14·5	16·6	17·0	17·0	14·8	14·6	13·6	14·7
Willow:								
On dry wood or residue, %:								
Total furfuraldehyde	14·49	14·12	15·30	15·68	8·37	9·40	9·27	8·81
Cross and Bevan furfuraldehyde	9·73	12·43	12·88	13·00	7·54	8·13	7·89	7·17
Chlorination loss	4·76	1·69	2·42	2·68	0·83	1·27	1·38	1·64
Cross and Bevan cellulose	59·93	73·09	72·18	70·58	70·45	71·56	70·00	68·13
On Cross and Bevan cellulose, %:								
Glucosan	74·8	73·6	72·5	71·4	83·4	82·4	82·5	83·7
Para-xylan	25·2	26·4	27·5	28·6	16·6	17·6	17·5	16·3

The figures for Cross and Bevan cellulose on dry wood or residue indicate the relative efficiencies of extraction of cellulosic and non-cellulosic materials. Thus, with both woods, increasing concentration of alcohol in the alcoholic-NaOH pretreatments diminishes the proportionate extraction of non-cellulose, whilst with mahogany the subsequent extraction with 4% NaOH further enhances the cellulose content of the residue, this extractant having here removed proportionately more non-cellulose than cellulose. With willow, on the other hand, the result of 4% NaOH extraction after alcoholic-NaOH pretreatment is to diminish the cellulose content of the residue; clearly, in this case, cellulose has been extracted more effectively than non-cellulose.

Again, no matter what pretreatment or extraction has been applied, chlorination always removes more furfuraldehyde-yielding material from the residue. Two possible explanations suggest themselves; either the various NaOH treatments increase the susceptibility of the para-xylan to the effects of chlorination

and sulphite extraction, or these NaOH treatments fail to remove the whole of the encrusting hemicelluloses. Whilst the first alternative is not to be overlooked, there is direct evidence to support the second; thus, Preece [1931] has shown that hot alkaline extraction of wood residues may continue to remove urono-pentosan after exhaustive extraction in the cold has failed to take any further appreciable amount into solution.

The variations found in the compositions of the various Cross and Bevan celluloses are of the first importance, and it may be remarked that a difference in para-xylan content exceeding 0·5 % between two samples may be considered significant. With mahogany, the compositions of the celluloses from the original wood and from the residues *hr*, hr_{10} and hr_{30} are substantially identical. Clearly this result by itself is most misleading; it must simply be implied that xylan and glucosan have been proportionately removed. It is apparent that, with this wood (and with willow also), hot alcoholic-NaOH removes glucosan more effectively than xylan, whilst with cold 4 % NaOH the reverse is true. Thus, the observed cellulose compositions depend only on the differing rates of extraction of the components; there is every reason to believe that both components are removed to greater or less extents by the various NaOH treatments applied. These results, especially in so far as they relate to hot alkaline extraction, may be compared with those recently obtained by Mackney [1940].

Discussion

The figures of Table 1 amply confirm the destructive effect of boiling alcoholic-NaOH on furfuraldehyde-yielding substances, and it is interesting to note that the magnitude of the loss, while but little affected for a given wood by the alcohol concentration, does vary considerably with different woods. Thus, for 50 % alcohol-1 % NaOH the losses for teak, mahogany and willow are respectively 4·2 % [Preece, 1940], 5·1 % and 9·0 % of the total furfuraldehyde, these being the figures when a pretreatment including alcohol extraction had been given. It appears that there may be a further source of loss. In Table 4

Table 4. *Hemicellulose yields*

	From mahogany		From willow	
Preparation	Calc. %	Actual %	Calc. %	Actual %
l_{10}	2·89	1·72	5·44	3·07
l_{30}	2·58	0·70	3·68	1·38
l_{50}	1·27	0·16	2·44	0·22

are given the calculated (Tables 1 and 2) and actual (Table 2) yields of hemicellulose soluble in alcoholic-NaOH. The yields obtained fall very short of those calculated and it is probable that depolymerization of some of the hemicellulosic material renders it incapable of precipitation by the usual means. More direct evidence of loss of hemicellulose is provided by noting that direct extraction of willow with 4 % NaOH gave a 10·64 % yield of hemicellulose; in no case with this wood did the combined yields of l_x and h_x approach this figure. On the other hand, the direct yield from mahogany is exceeded by $(l_{10}+h_{10})$ and by $(l_{30}+h_{30})$. There can be no doubt that the alcoholic-NaOH treatments do cause the dissolution of more hemicellulose than would be the case with direct cold extraction alone, but whether this increased dissolution is reflected in the yields obtained depends entirely on the susceptibility to degradation of the material concerned.

The evidence is complete that hemicelluloses without exception contain material (cellulosan) which is resistant to chlorination and also material (encrusting hemicelluloses) which is removed by this treatment. Indeed, in the case at least of the willow preparations, the amount of cellulosan present probably predominates. Table 3 indicates that the residues of the lr_x series yield more crude cellulose than the corresponding members of the series hr_x. This can only be the case if cellulosic material is removed more completely than non-cellulose (including encrusting hemicelluloses) by the intervening NaOH extraction. In fact, with the figures given, the cellulosan passing into the hemicellulose extract must exceed twice the amount of non-cellulosan, not all of which need be encrusting hemicellulose. Certainly, however, the differences between willow pairs are not significant except in the case of lr_{10} and hr_{10}; nevertheless, this scarcely affects the above argument. The influence of direct cold extraction with 4 % NaOH on furfuraldehyde-yielding cellulosan materials is shown by a comparison of the para-xylan figures for the cellulose of willow wood and that of the residue *hr* (Table 3).

Hot extraction likewise gives mixtures of hemicelluloses, but the tendency here is towards increased removal of non-pentosan. The relatively low figures for 'lignosaccharide' furfuraldehyde thus appear to be largely accounted for by relatively high glucosan contents, though there may also be differences in uronic anhydride, a point which has not here been investigated. 'Lignosaccharide', therefore, does not differ from hemicelluloses extracted in the cold, except in the proportions of the various components of the mixture. It may be noted that hot extraction, which favours the dissolution of glucosan, also gives fractions showing higher lignin figures when examined by the Schwalbe method; a difference persists even when careful Cu purification is applied. It is possibly worthy of investigation whether these fractions contain high proportions of material either (i) chemically associated with lignin or (ii) of such modified properties as to give products resistant to 72 % H_2SO_4-18 % HCl treatment.

It is abundantly clear therefore that the methods of hemicellulose preparation at present employed give mixed products. It has been claimed on various occasions to separate preparations into fractions that were 'chemically and physically distinct' [Norris & Preece, 1930]; exception has been taken to such claims, but it has never been implied that the fractions were homogeneous. Again, Preece [1931] showed that the analysis of box-wood hemicellulose could be interpreted as indicating a relatively simple composition; but here again there was no suggestion that a simple 'pure' product was, in fact, concerned. The dangers of the problem are further indicated by comparing the results for maize-cob hemicelluloses obtained by Preece [1930] with those for the same materials of Angell & Norris [1936]. The latter authors show that, by careful control of *p*H, Preece's fractions A_1, B_1 and C_1 can be precipitated as a single fraction. However, A_1, B_1 and C_1 show distinct differences in chemical composition, even though the partial separation achieved may have been fortuitous; evidence of the possibility of even a partial fractionation would appear to be more informative than the demonstration that, under other conditions, separation is avoided. Further, in relation to these maize-cob products, it now seems certain that their chemical analysis is to be accounted for on the assumption that the products of hydrolysis are pentose, uronic acid and glucose.

SUMMARY

1. Loss of hemicellulose material as a result of boiling alkaline pretreatment is confirmed.

2. Hot alkaline extraction of woods favours the dissolution of non-pentosan; extraction in the cold favours the dissolution of xylan.

3. Hemicellulose preparations contain materials derived both from the encrusting hemicelluloses and from the cellulose fraction; the relative proportions of the components of the mixture vary with the conditions of extraction and with the raw material.

4. The mixed nature of hemicellulose preparations is stressed.

The author's best thanks are due to his colleagues, Messrs J. W. Ingham and James Mathieson, for valued assistance and advice.

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69. HISTIDINE IN URINE. 2

By E. RACKER

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(*Received 31 May 1941*)

In a previous paper [1940] a method has been described for quantitative estimation of histidine in urine. Numerous estimations have been carried out and values corresponding to 2–80 mg. histidine monohydrochloride per 100 ml. have been found in normal urines. These figures were unexpectedly high, and so it was decided to investigate further the nature of the substance or substances responsible for the bromine colour test.

Experimental

1. *Purification of the substance giving the bromine colour test*

Urine, in portions of 200–300 ml., was shaken with 20 g. purified animal charcoal for 25 min. The urine was then filtered through a Büchner funnel and the filtrate poured into a separate flask. The charcoal, which remained in the Büchner funnel, was washed with $N/10$ H_2SO_4 until all the histidine adsorbed by the charcoal was eluted and the last washing became negative to the colour test. The first urine filtrate, which was kept separate, was again shaken with charcoal, filtered and the filtrate kept separate, the charcoal being washed with $N/10$ H_2SO_4. This procedure was repeated until the original urine was free from histidine. The combined washings were then evaporated *in vacuo* to about 100 ml. and treated with hot saturated baryta until neutral to phenol red. The precipitated barium sulphate was centrifuged down, the supernatant fluid decanted, the precipitate washed with hot distilled water and again centrifuged. The combined supernatant fluids were evaporated *in vacuo* to about 50 ml. and 5 ml. N H_2SO_4 were added. To this an excess of $AgNO_3$, dissolved in distilled water, was added (6–8 g. were usually required). After precipitation was completed, the mixture was filtered, the precipitate of silver chloride being washed with distilled water. The clear filtrate was brought to *p*H about 8·0 with $2N$ NaOH and left overnight in the refrigerator. The precipitate was filtered off and taken up in distilled water. It was then acidified with dilute H_2SO_4 and decomposed with H_2S for about 12 hr. The mixture was filtered, the precipitate washed and the combined filtrates evaporated *in vacuo* to a low bulk. This purified solution was analysed by the bromine reaction and diluted until it corresponded with a 1 % solution of histidine monohydrochloride. The purified material was used for further investigation.

2. *The biological breakdown of the substance in urine*

It has been shown by Edlbacher [1926] that liver contains an enzyme, histidase, which breaks down histidine. The activities of this enzyme in breaking down histidine and the substance excreted in urine have been compared.

The liver was passed through a Latapie mincer, then ground with sand and suspended in $M/15$ Na phosphate buffer of *p*H 8·0, so that 4 ml. of the suspension contained 500 mg. of liver brei. The suspension was centrifuged for 20 min.,

the supernatant fluid decanted and used as enzyme solution. 4 ml. were used for each experiment. The activity of the enzyme was noted, comparing the breakdown of 10 mg. histidine monohydrochloride with that of the corresponding amount of the purified urine solution over varying periods. After incubation in a water bath at 40° the mixture was precipitated with 5 ml. of

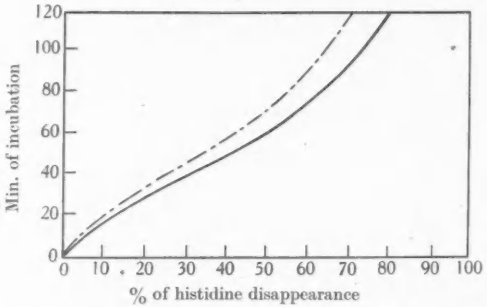

Fig. 1. Rat liver histidase activity. —— Histidine HCl. —·— Urine histidine.

20 % trichloroacetic acid and centrifuged; the supernatant fluids were decanted and the precipitate twice washed with distilled water. The fluids were collected and made up to a known volume. The clear solutions were analysed by the bromine reaction and the results obtained are shown graphically in Fig. 1.

3. *Pharmacological test of the substance in urine*

Hanke & Koessler [1922; 1924] studied the formation of histamine from histidine by various types of *Bact. coli*. Recently Gale [1940] has followed this breakdown under more favourable conditions in manometric experiments. Using suspensions of 'resting bacteria' [Quastel & Whetham, 1924] he measured the activity of the decarboxylase for various amino-acids.

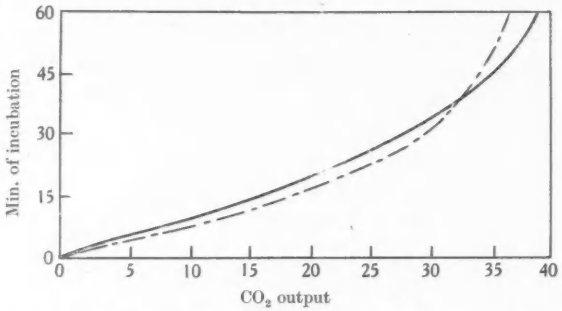

Fig. 2. Decarboxylation of histidine. —— Histidine HCl. —·— Urine histidine.

The decarboxylation of the substance in urine has been followed by this method, using a standard histidine solution as control. After the manometric experiments were concluded the solutions were tested pharmacologically.

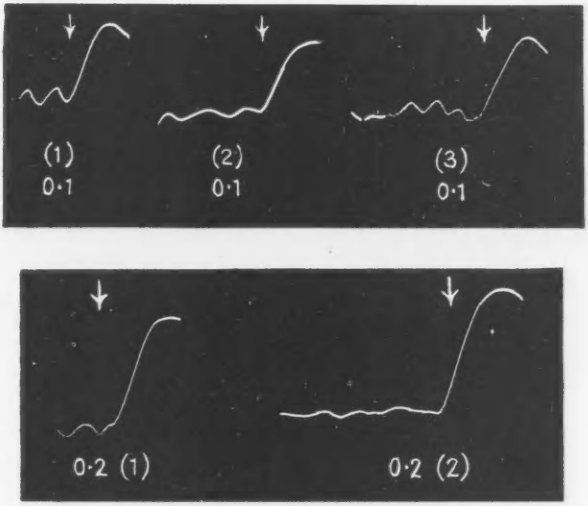

Fig. 3. Fowl caecum. (1) 0·1 μg. histamine HCl. (2) 0·1 μg. histamine from histidine HCl. (3) 0·1 μg. histamine from urine histidine. Lower figure: 0·2 μg. histamine HCl.

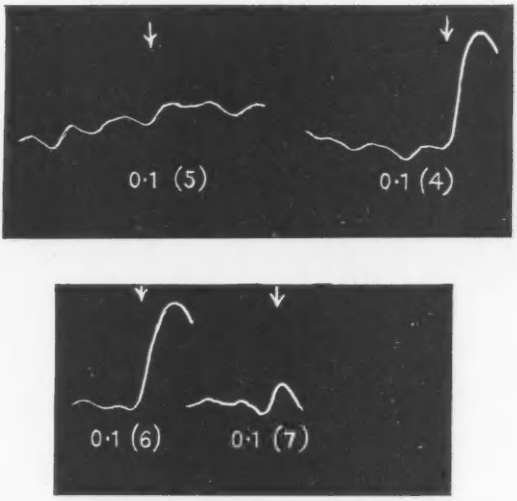

Fig. 4. Fowl caecum. (4) 0·1 μg. histamine from urine histidine. (5) 0·1 μg. histamine from urine histidine + histaminase. (6) 0·1 μg. histamine HCl. (7) 0·1 μg. histamine HCl + histaminase. All incubated for 24 hr. at 37° and then tested.

The respiratory experiments have been carried out under the conditions laid down by Gale. The bacteria (*Bact. coli Esch.* Nat. Inst. of Type Cultures, no. 86) were grown in 2% glucose broth at 27° for 18 hr., centrifuged, well washed and suspended in distilled water. The dry weight was determined in each case by a photoelectric turbidimeter. 1 ml. of the bacteria suspension was pipetted into each Warburg vessel with 1 ml. of phthalate buffer (*p*H 4·0) and 0·7 ml. distilled water. 0·3 ml. of the histidine or urine solution was placed in the side cup. After equilibration in the thermostat at 30° the histidine was tipped in. The results were recorded graphically (Fig. 2).

After incubation the bacteria were centrifuged off, the clear solutions neutralized and diluted with Tyrode solution to a concentration of histamine 1 μg./ml. according to the theoretical amounts of histamine formation to be expected from the CO_2 output.

The solutions were then tested on the isolated caecum of the fowl [Barsoum & Gaddum, 1935]. The gut was suspended in a small water bath containing 8 ml. of a Tyrode glucose solution, kept at 37° and continuously aerated with oxygen. The amount of fluid tested did not exceed 0·2 ml. The contractions of the gut were recorded on a slowly rotating smoked drum and typical results are given in Fig. 3.

Histaminase (Torantil Bayer), which breaks down histamine, acts in the same way on the histamine produced from normal urine (Fig. 4).

4. *Estimation of histidine by nitranilic acid*

Block [1940] has shown that histidine is quantitatively recovered from solutions as nitranilate and he has used this for estimation of the amino-acid. This method was applied to the purified urine solution and quantitative recoveries, corresponding to the bromine colour test, have been obtained. The experimental procedure was as follows.

The purified urine solution was first treated with an excess of 95% alcohol and centrifuged. The precipitate was dissolved in distilled water, reprecipitated with alcohol and centrifuged. This process was repeated until the precipitate was free of histidine. The collected supernatant fluids were evaporated *in vacuo* to a few ml. and an equal amount of absolute methyl alcohol was added. To this the nitranilic acid, dissolved in distilled water, was added in excess. The mixture was left in the refrigerator overnight, and the centrifuged precipitate was then dried to constant weight. The result obtained is given in Table 1.

Table 1

	Histidine present mg.	Dry weight of precipitate mg.	Recovery %
Histidine HCl	40	71·8	97·5
Urine histidine	40	76·8	104·2

Discussion

The experimental evidence presented above indicates that the greater part of the substance in urine reacting with bromine is histidine. Recent work on the subject [Sakaguchi, 1940; Langley, 1941] is in agreement with this conclusion.

Tschopp & Tschopp [1938] have investigated the excretion of histidine in various diseases, but as they were working with a qualitative method the results appear not to be conclusive.

A small number of quantitative histidine estimations have been made on patients with malignant tumours, relating the histidine excretion to the specific gravity and urea excretion; so far normal figures have always been obtained.

Two pathological conditions are of special interest with regard to histidine metabolism.

1. Experimental shock, produced by a procedure described elsewhere [Quastel & Racker, 1941]. Measurements of histamine formation *in vivo*, and by tissues anaerobically *in vitro*, have not, however, been convincing enough to justify any conclusion.

2. *Bact. coli* infection. Considering the large amounts of histidine excreted normally and the relatively small amount of histamine necessary to exert a pharmacological effect, investigation of the fate of urinary histidine seems to be indicated. The acidity of the urine prevailing in *Bact. coli* infection of the urinary tract should be a favourable factor for histamine formation. Because of lack of suitable clinical material this investigation has not yet been carried out.

It may be noted that in severe cases of toxaemia of pregnancy histamine has been found and isolated from urine by Kapeller-Adler [1941], thus indicating an altered histidine metabolism in this condition.

Summary

1. The procedure of purification of the substance in urine, giving rise to the bromine colour test, is described.

2. The substance is broken down by liver histidase.

3. The decarboxylation of the substance has been followed in manometric experiments and the fluids have then been tested for their action on the isolated fowl's gut. If incubated with histaminase the effect on the gut is nullified.

4. The nitranilic acid precipitation method has been applied to the urine substance.

5. It is concluded that the colour produced by the bromine reaction is due to histidine. The clinical significance of this is discussed.

The author wishes to express his indebtedness to Dr J. H. Quastel for his interest and advice throughout this work.

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70. A METHOD FOR THE MICRO-ESTIMATION OF IRON IN BIOLOGICAL MATERIALS

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(*Received 23 April 1941*)

A METHOD for the estimation of very small quantities of iron in biological materials has been in use in this laboratory for some years. It is based upon the reaction of ferrous Fe with $\alpha\alpha'$-dipyridyl described by Hill [1930]. The method has proved so useful for the study of Fe in biological materials of all kinds, such as bacterial cultures, media and tissues of animal origin, that it seems worthy of wider use. When the concentration of the red dye produced by the reaction of ferrous Fe and $\alpha\alpha'$-dipyridyl is measured colorimetrically it is possible to estimate 0·2 μg. Fe per ml. of solution under examination. The sensitivity of the method is approximately the same as that of the thiocyanate method for the estimation of ferric Fe. The presence of Cu, Zn and other metals does not interfere with the test when the quantities of these elements are comparable with that of the Fe [Hill, 1930]. Cu gives a blue colour, but this does not interfere with the estimation of Fe, even when present in amounts up to 1 mg./ml., as a green light filter is used in the colorimeter and the green light is not absorbed by the blue colour due to the Cu. Zn forms a colourless compound with $\alpha\alpha'$-dipyridyl, but this does not affect the Fe reaction when the reagent is present in excess. Although by theory three molecules of $\alpha\alpha'$-dipyridyl combine with one atom of Fe (in molar equivalents, 468 g. of $\alpha\alpha'$-dipyridyl to 56 g. of Fe), in practice it has been found best to use ten times more dipyridyl than the theoretical amount. Since the red complex produced in the presence of excess $\alpha\alpha'$-dipyridyl is dissociated over a range of *p*H 3·5–8·5, it is satisfactory to estimate the colour at about *p*H 6. In our method the standard solutions have a *p*H of 6·3. Hill has shown that Fe may be estimated in many biological materials without previous ashing if precautions are taken to prevent the adsorption of the red iron dipyridyl upon insoluble proteins. We have preferred always to ash the materials by the usual wet ashing process, using perchloric acid, HNO_3, or H_2O_2, instead of selenium or catalytic copper methods, for oxidation. The red colour of the complex ion is formed only in the presence of ferrous Fe so that reduction is necessary after ashing the material. This is effected by sulphurous acid formed from sodium sulphite in the presence of HCl. At present $\alpha\alpha'$-dipyridyl is not commercially available but it may be prepared in several ways. Blau [1888] prepared it first, from copper picolinate, and Hill describes a method based upon this reaction. Hein & Retter [1928] devised a simpler method of preparation by the oxidation of pyridine in a closed tube. We have found it convenient to use the method of Wibaut & Overhoff [1928], in which 2-bromopyridine is boiled with cymene in the presence of copper powder for 1½ hr. The cymene is then distilled off in steam, the residue made alkaline and finally the $\alpha\alpha'$-dipyridyl is distilled. The product is purified by recrystallizing from 20% alcohol.

Method

Reagents

Iron-free HCl. 20 % HCl is distilled in an apparatus made entirely of pyrex glass. Two strengths of the acid are prepared from the distillate, *N* and *N*/10; these are standardized and should be within 1 % of the nominal strengths.

αα'-Dipyridyl reagent. An accurately weighed amount of the material is dissolved in sufficient 0·1 *N* HCl to make a 1 % solution. This solution is quite stable but should be stored in a white or pyrex glass bottle which has been filled with conc. HCl and allowed to stand for some days, and then repeatedly rinsed in glass-distilled water. Under these conditions the reagent should show no perceptible pink colour.

Sodium sulphite solution. A 20 % solution is prepared freshly as required from A.R. sodium sulphite crystals, $Na_2SO_3, 7H_2O$.

Standard iron solution. A stable stock solution of Fe is prepared from ferric ammonium sulphate by dissolving 0·4318 g. in a little water, adding 100 ml. of *N* iron-free HCl and making up to 1 l. with glass-distilled water. This solution contains 50 μg. Fe/ml.

Standard colours

Standard colours are prepared containing from 0·2 to 10 μg. Fe/ml. The standard solution when diluted five times contains 10 μg. Fe/ml. Appropriate dilutions are made from this by using a micrometer syringe or burette and the

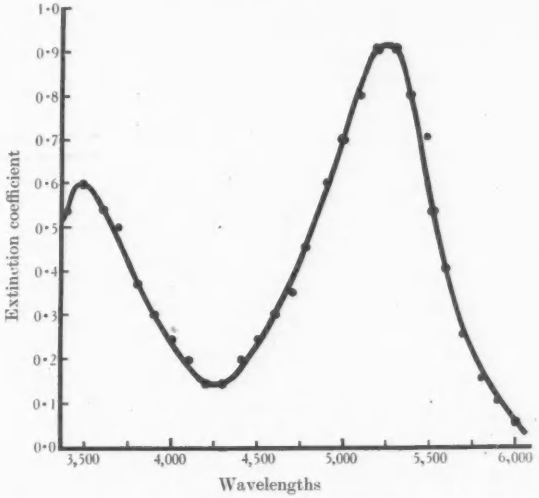

Fig. 1. Spectrographic absorption curve of iron dipyridyl solution.

volumes adjusted to 1 ml. with distilled water. It is convenient to dilute the solutions in $3 \times \frac{3}{8}$ in. tubes calibrated at 1 ml. To each tube is now added 0·4 ml. of the dipyridyl reagent followed by 0·5 ml. of 20 % sodium sulphite solution and 0·1 ml. of *N* HCl. The red colour reaches a maximum in 15 min. and is permanent; it may be compared optically or photoelectrically. The peak of the

absorption of the red dye occurs at a wave-length of 5250 Å. In Fig. 1 is reproduced an absorption curve of the red colour of iron dipyridyl, which was kindly furnished by Dr Foster of the Wellcome Chemical Works, Dartford. If a powerful light source is available a Wratten green filter no. 62 or Ilford 'Mercury Green' type of filter is most suitable, but if this is too dense a filter no. 61 is almost as efficient and transmits far more light. The usual convention of plotting the concentration against the logarithm of the percentage transmission is followed. A typical calibration is shown in Fig. 2, derived by using a photoelectric colorimeter employing a gas-filled caesium cell. If the filtered light is truly monochromatic, or nearly so, and is complementary in colour to the absorption band

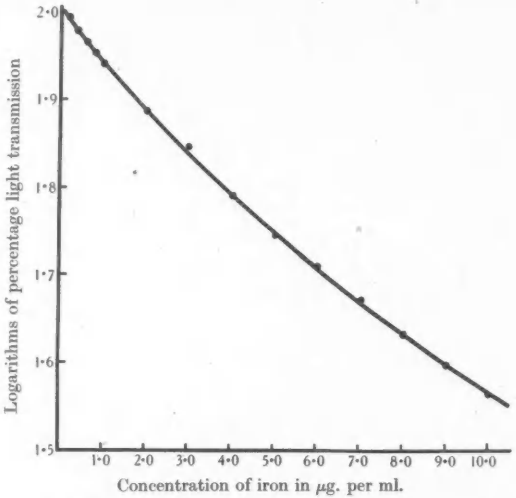

Fig. 2. Calibration curve for photoelectric colorimeter.

of the solution, this curve will be linear. This is rarely the case unless the filter used is very dense or the solutions being compared are pale in colour. The calibration curve as shown in the figure is, however, quite satisfactory in practice. If the range of standard solutions of Fe used in the calibration extends only to 5 μg./ml. the curve is virtually linear when plotted in the same way. Using a colorimeter employing a selenium cell the sensitivity is lower but a suitable calibration curve can be prepared in the same way over a wide range of Fe concentration. Although this curve is less reliable at lower values of Fe concentration it is equally practicable, and is particularly useful when the range of the concentrations of Fe is large.

Estimation of Fe in samples

For tissues or foodstuffs we have found it convenient to weigh out accurately an amount of material estimated to contain between 10 and 200 μg. Fe and ash by the wet ashing method described by Eden & Green [1940] using H_2SO_4 and perchloric acid. With about 1 g. or less of tissue it is unnecessary to use HNO_3 as an additional oxidizing agent. The excess acid is neutralized with solid Na_2CO_3 (A.R.) until there is no further effervescence. The neutral solution

is then reacidified with HCl to about *p*H 4; the exact value is not vital, owing to the buffering effect of the sulphite in the subsequent stages of the estimation. The solution is then diluted to 50 ml. in a standard flask with glass-distilled water. For the preparation of the coloured solution 1 ml. of the liquid containing Fe is added to 0·4 ml. of the dipyridyl reagent in one of the small tubes and this is followed by 0·5 ml. of sodium sulphite solution and 0·1 ml. of *N* HCl, as in the case of the standard colours. The mixture is then shaken and the red colour developed is read upon the colorimeter after an interval of 15 min. The concentration of Fe in the solution is then calculated from the calibration curve. In view of the sensitivity of the method it is necessary to prepare control tubes covering the chemicals used in the entire process. The value obtained for the blank is then subtracted from that obtained in the estimations. If the red colour developed is too deep for the range of the colorimeter the solution may be diluted with water until a convenient reading is obtained. Where the Fe content is known to be high by previous estimations it is more convenient to dilute the solution obtained after ashing and neutralization.

Summary

1. A method of estimating small amounts of Fe in biological materials is described. The method is based upon the red complex formed when $\alpha\alpha'$-dipyridyl reacts with ferrous Fe.

2. Amounts of Fe as small as 0·2 μg./ml. can be estimated colorimetrically or optically.

3. An outline is given of methods for preparing $\alpha\alpha'$-dipyridyl, since this is not available commercially.

I wish to take this opportunity of thanking Mr R. Sellar for the technical assistance he has given in the chemical work associated with the method.

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71. BIOLOGICAL SYNTHESIS OF OXALOACETIC ACID FROM PYRUVIC ACID AND CARBON DIOXIDE

2. THE MECHANISM OF CARBON DIOXIDE FIXATION IN PROPIONIC ACID BACTERIA[1]

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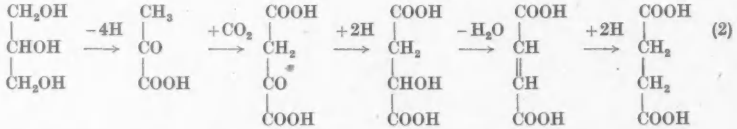
(*Received 4 April 1941*)

Wood & Werkman [1936, 1; 1938] found that propionic acid bacteria utilize CO_2 when glycerol is present and that the CO_2 fixation is coupled with the formation of an equivalent amount of succinic acid. These observations are summarized by equation (1):

$$CH_2OH.CHOH.CH_2OH + CO_2 = COOH.CH_2.CH_2.COOH + H_2O. \quad (1)$$

Recently, Wood & Werkman [1940] suggested the following mechanisms for this reaction:



(2)

This scheme is supported by several experimental observations. Wood *et al.* [1937] found that glycerol in accordance with (2) can be converted into pyruvic acid. Wood *et al.* [1940] showed, with the help of the isotope C^{13}, that the fixed CO_2 appears in fact in the carboxyl groups of succinic acid. The experiments reported in this paper were mainly undertaken to supply further evidence in support of scheme (2).

The organism

A culture of *Bacterium acidi propioni* obtained from the National Collection of Type Cultures (No. 4759) showed a very feeble fermenting capacity. The organism[2] grew vigorously under aerobic conditions (on the surface of agar), but it failed to grow anaerobically on glucose-lactate-yeast water, or on whey. Suspensions of washed bacteria, grown on agar, did not attack glucose, lactate or glycerol under anaerobic conditions; pyruvate was slowly decomposed. Attempts to recover the original fermenting power by transferring the organism to liquid media (glucose-lactate-yeast water, whey) were unsuccessful. Even after 2 months no growth or fermentation was perceptible. The Curator of the Collection informed us that the organism had been cultivated for 5 years on agar slopes. This may account for the inability to ferment, for Jensen [1931,

[1] A preliminary report appeared in *Chem. and Ind.* **59**, 849 (1940).

[2] This is the organism recently used by Quastel & Webley [1939; 1941]. We can confirm the effects of vitamin B_1 described by Quastel & Webley with this atypical bacterium, but we obtained no vitamin effects with the six strains of propionic acid bacterium used in the present paper.

p. 47] states 'when propionic acid bacteria have become accustomed to aerobic conditions, their fermentative powers are gradually weakened'.

The organism used in the greater part of this work was isolated from Gruyère cheese obtained at a local shop. Material from the 'eye' of the cheese was spread over an agar plate of lactate-yeast water and incubated at 25° in an anaerobic jar filled with CO_2. Among several organisms a typical propionic acid bacterium was obtained. Its properties are as follows:

(1) Very short, non-motile, gram-positive rods, non-spore forming, assuming involution forms in old cultures, or when grown aerobically at 37°. Agar stab culture: abundant stab growth, moderate surface growth.

(2) Lactate is rapidly fermented under anaerobic conditions, yielding 25–30 % CO_2 and 90–100 % fixed acids. Among these propionic acid was identified as mercurous salt [Musicant & Kaszuba, 1939].

(3) Glycerol is slowly fermented with the fixation of CO_2 and formation of succinate, as described by Wood & Werkman.

(4) Acid is produced from glucose, lactose, galactose, mannose, fructose, arabinose.

(5) Neither acid nor gas is produced from starch, sucrose, xylose.

(6) A powerful catalase is present.

The properties listed under (1), (2) and (3) characterize the organism as a *Propionibacterium*. The other properties are identical with those of the species *Propionibacterium Shermanii* [Sherman, 1920; van Niel, 1928; Werkman & Brown, 1933].

During the later stages of this work we received five strains of propionic acid bacteria of the American Collection of Type Cultures through the courtesy of Dr J. G. Davis, National Institute for Research in Dairying, University of Reading. They were *P. Freudenreichii*, *P. Shermanii*, *P. Jensenii*, *P. Thönii* and *P. rubrum*. Some experiments were repeated with these organisms.

Most of the experiments reported in this paper were carried out with suspensions of washed cells. The medium used for the large scale preparation of bacterial suspensions contained the following ingredients:

200 ml. yeast water (200 g. dried brewer's yeast and 800 ml. tap water) heated in the autoclave (120°, 30 min.) and filtered. The liquid contained about 3·5 % dry matter.

200 ml. phosphate buffer (0·1 *M*, *p*H 7·4).

25 ml. tryptic casein digest [Gladstone & Fildes, 1940].

40 ml. 50 % sodium lactate.

500 ml. tap water.

25 g. agar.

Sterile medium was poured into large sterile petri dishes (15 cm. diameter) and these were inoculated with 3–4 ml. of a liquid culture (the above medium without agar). The petri dishes were placed in a vacuum jar. The air pressure in the jar was reduced on the water pump to 15–20 mm. Hg and 400–500 mm. CO_2 were then admitted. The temperature of the incubator was 34°. After 4–5 days the cells were washed off the agar with distilled water, centrifuged, resuspended in water, centrifuged again and finally suspended in a small volume of water. This 'stock suspension' was kept in the refrigerator. In the case of *P. Shermanii* the yield was about 200–250 mg. dry cells per petri dish. For metabolic experiments the stock suspension was diluted with solutions of $NaHCO_3$ or phosphate. The concentrations used are stated in the tables.

The manometric and analytical methods were the same as those described in previous papers from this laboratory. All experiments were carried out at 30°.

Metabolism of oxaloacetate in Propionibacterium Shermanii

The metabolism of oxaloacetate was investigated in order to see if oxaloacetate can react to form malate, fumarate and succinate as predicted by scheme (2). The suspension contained 14 mg. cells per ml. 0·031 *M* $NaHCO_3$ solution. Four conical Warburg cups were used and 4 ml. suspension were measured into each cup. The side-arms contained 0·5 ml. 0·2 *M* oxaloacetate, the centre chamber yellow phosphorus and the gas space CO_2. The CO_2 production was measured over a period of 22 hr. Two cups were removed from the bath after 3 hr. for the determination of succinate and of fumarate and malate [Krebs, Smyth & Evans, 1940]. The remaining cups were left in the bath until the CO_2 production had almost stopped and succinate, fumarate and malate were then determined. The results are shown in Table 1.

Table 1. *Metabolism of oxaloacetate in* Propionibacterium Shermanii

For experimental conditions see text; the data refer to 4 ml. suspension.

Time hr.	μl. CO_2 formed	μl. succinate formed	μl. fumarate + malate formed
1	335	—	—
2	585	—	—
3	920	654	322
4	1024	—	—
5	1118	—	—
21	1640	—	—
22	1655	908	0

Table 2. *Formation of bicarbonate from oxaloacetate in* Propionibacterium Shermanii

3 ml. suspension containing 38 mg. bacteria; 5% CO_2 in N_2; initial amount of bicarbonate in 3 ml.: 878 μl.; concentration of oxaloacetate: 0·0061 *M*; period of incubation 6 hr.

	μl.
Free CO_2 evolved	239
Final amount of bicarbonate	1030
Increase in bicarbonate	152

The quantity of free CO_2 (not including HCO_3') evolved amounted to 74% of the oxaloacetate added. Fumarate and malate appeared in the earlier stages, but disappeared later. The amount of succinate increased with time and reached about 40% of the added oxaloacetate. It should be remembered that the decarboxylation of oxaloacetate does not cause an increase in CO_2 pressure since in this reaction bicarbonate is formed.

The formation of bicarbonate was measured in another experiment [for methods see Krebs, Eggleston, Kleinzeller & Smyth, 1940] and it was found (Table 2) that the quantities of bicarbonate formed are somewhat smaller than those of free CO_2.

The data observed agree approximately with the following scheme of oxaloacetate fermentation:

$$3 \text{ oxaloacetate} = 1 \text{ succinate} + 2 \text{ acetate} + 2HCO_3' + 2CO_2.$$

The yields of succinate and of free CO_2 are greater than expected (40·5% succinate instead of 33·3%; 74% CO_2 instead of 66·6%), while the yield of bicarbonate is below the calculated quantity. These discrepancies are probably due to other reactions of oxaloacetate. In the above scheme the conversion of

2 mol. of oxaloacetate into acetate (via pyruvate) is the equivalent of the reduction of a third oxaloacetate molecule to succinate. Various experiments suggest that oxaloacetate can also be reduced at the expense of other oxidative processes in which either intracellular substances, or acetate (see later) undergo oxidation and this would account for the experimental deviations from the theory.

Experiments with mesoxalate and α-ketoglutarate showed that these nearest homologues of oxaloacetate are not metabolized by washed suspensions of *P. Shermanii*. The organism thus possesses a highly active enzyme system which specifically ferments oxaloacetate. The existence of this system strongly suggests that oxaloacetate is a normal metabolite in *P. Shermanii*.

Metabolism of fumarate and malate in Propionibacterium Shermanii

(1) *Presence of fumarase.* Fumarate is rapidly converted into *l*(−)-malate as shown in the following polarimetric experiment: 50 ml. 0·1 *M* fumarate (=5 m.mol.), 20 ml. 0·1 *M* phosphate buffer, *p*H 6·8; 14 ml. bacterial suspension containing 331 mg. dry cells and 2·5 ml. propyl alcohol were incubated at 30°. At various intervals 15 ml. were removed, mixed with 1 g. uranyl acetate and 0·5 ml. glacial acetic acid and heated for 10 min. in a boiling water bath. After cooling and filtering, the rotation of the solution was determined in a 1 dm. tube. The readings were: after 60 min. −0·59°; 120 min. −1·40°; 360 min. −2·47°. Experiments with pure *l*(−)-malic acid showed that the specific rotation was about 485° under the conditions used. The quantity of *l*(−)-malic acid formed (m.mol.) was therefore after 60 min. 0·813; 120 min. 1·93; 360 min. 3·40. In terms of the usual metabolic quotients the rate of malic acid formation is for the first hour 55 μl. per mg. cells per hour. The occurrence of fumarase in propionic acid bacteria has also been reported by Erkama [1938].

(2) *Reduction to succinate.* A suspension containing 9 mg. cells per ml. 0·04 *M* $NaHCO_3$ solution was used and 0·25 ml. 0·2 *M* fumarate (=1120 μl.) was added to 4 ml. suspension. Otherwise the conditions were the same as those described in the analogous experiments with oxaloacetate. The evolution of CO_2 (including CO_2 liberated from $NaHCO_3$) was 99 μl. in 1 hr.; 184 μl. in 2 hr.; 475 μl. in 18 hr. After 18 hr. the solution contained 1032 μl. succinate. Thus the yield of succinate, in terms of added fumarate was 95 %. The nature of the oxidative equivalent for reduction of fumarate has not been investigated in detail. Obviously intracellular substances serve as substrates for the oxidative reactions. That oxidizable substances are present is shown by the O_2 uptake of washed cells in the absence of added substrate (Table 3).

Table 3. *Rate of respiration of* P. Shermanii

The data refer to 3 ml. suspension containing 14·4 mg. bacteria. Medium 0·04 *M* phosphate buffer, *p*H 6·8; gas, air; 30°. Substrate concentration: 0·02 *M*; no increase was observed on addition of glycollate, oxalate, formaldehyde, ethyl alcohol.

Substrate added		*d*(−)-Lactate	Glycerol	Succinate	*l*(−)-Malate	Acetate	Propionate	Formate	Glucose
μl. O_2 absorbed after									
20 min.	56	122	113	68	55	85	86	88	118
40 min.	86	330	260	115	93	167	182	182	232
60 min.	124	506	412	158	138	240	285	255	358
Q_{O_2}	8·6	35·2	28·6	10·9	9·6	16·7	19·8	17·7	24·8

(3) *Experiments with* l(−)-*malate.* Fumarate and *l*(−)-malate behaved identically in *P. Shermanii*, as may be expected if a powerful fumarase is present;

d(+)-malate was not metabolized. This is worthy of note since the stereoisomerides of lactate were both fermented at the same rate.

Rate of succinate formation from various substrates

If pyruvate, oxaloacetate and fumarate are intermediates in the formation of succinate from glycerol, as suggested by scheme (2), the succinate formation from these substrates should be at least as rapid as the succinate formation from glycerol. Table 4 shows that such is the case. Although the yield of succinate is much lower when lactate and pyruvate are the substrates, the *rate* of succinate formation from these substances is of the same order as that from glycerol.

Table 4. *Rate of succinate formation from various substrates*

39 mg. bacteria in 4 ml. 0·03 *M* $NaHCO_3$. Initial substrate concentration 0·02 *M*; gas phase CO_2; period of incubation (1 hr.).

Substrate	Glycerol	*dl*-Lactate	Pyruvate	Oxalo-acetate	Fumarate or *l*(−)-malate
µl. CO_2 and acid	96	676	532	352	131
µl. succinate	73	95	83	177	372

Fermentation of glycerol

(1) *Formation of fumarate.* In the case of *Bact. coli* it has been possible to demonstrate a simultaneous formation of succinate and fumarate from pyruvate [Krebs & Eggleston, 1940]. In experiments with *P. Shermanii*, using glycerol as a substrate, we obtained similar results when an excess of glycerol was present. Details of the experimental conditions are given in Table 5. The yields of fumarate

Table 5. *Formation of fumarate from glycerol in* P. Shermanii

64 mg. bacteria in 6 ml. 0·04 *M* $NaHCO_3$: 5% CO_2+N_2; 2% glycerol; 21 hr.

	Exp. I	Exp. II
µl. CO_2 formed	1540	1380
µl. succinate formed	586	690
µl. fumarate formed	64	62

are rather low, presumably because of the rapid reduction of fumarate in the presence of glycerol (see the following paragraph). The simultaneous formation of fumarate and succinate can be taken as evidence in support of scheme (2) [see Krebs & Eggleston, 1940].

(2) *Effects of fumarate and oxaloacetate on the fermentation of glycerol.* When fumarate and glycerol are added together to washed suspensions of *P. Shermanii* the rate of acid and CO_2 production is 2–3 times greater than expected from experiments in which the substrates are added alone (Table 6). The rate of

Table 6. *Effect of glycerol and fumarate on the acid and CO_2 production by* Propionibacterium Shermanii

36 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.) ...	None	Fumarate (0·02 *M*)	Glycerol (0·02 *M*)	Fumarate (0·02 *M*), glycerol (0·02 *M*)
µl. CO_2 evolved after 20 min.	13	47	30	87
40 min.	30	93	65	212
80 min.	39	142	98	440
200 min.	49	228	236	1385
280 min.	83	444	314	1980
µl. succinate formed (280 min.)	—	1556	346	2008

succinate formation is also increased and the data suggest that the following reaction occurs in the presence of glycerol and fumarate:

glycerol + fumarate → oxidation products of glycerol + succinate.

This scheme, however, does not account for all the observations. When small quantities of fumarate are added the increased CO_2 output and succinate formation is equivalent to a multiple of the fumarate added (Table 7). Thus 112 μl.

Table 7. *Effect of oxaloacetate and fumarate on the succinate formation in the presence of glycerol*

26 mg. bacteria in 4 ml. 0·04 *M* $NaHCO_3$; CO_2.

Substrate added (final conc.)	... ... 0·1 *M* glycerol	0·1 *M* glycerol, 0·00125 *M* oxaloacetate (=112 μl.)	0·1 *M* glycerol, 0·00125 *M* fumarate (=112 μl.)
μl. CO_2 formed after 100 min.	106	322	280
280 min.	302	730	562
22 hr.	1100	1756	1488
μl. succinate formed	374	814	714
Extra succinate (due to addition of oxaloacetate or fumarate), μl.	—	440	340

fumarate cause an increase in CO_2 output of 388 μl. and in succinate formation of 340 μl. The effect of fumarate, therefore, is catalytic. From what is known about the metabolism of fumarate in other cells it is very probable that the catalytic effect is associated with hydrogen transport; it might be due either to the system succinate ⇌ fumarate, or the system fumarate ⇌ oxaloacetate. The first possibility, however, can be excluded, for if succinate were reversibly dehydrogenated it should be possible to replace fumarate by succinate, and this is not the case. Addition of succinate has no effect on the rate of acid production from glycerol and the added succinate is quantitatively recovered from the solution (Table 10). These results show that the system succinate ⇌ fumarate is not a hydrogen carrier under *anaerobic* conditions. Apparently O_2 cannot be replaced by other physiological oxidizing agents. On the other hand oxaloacetate can replace fumarate (Table 7) and the catalytic effect with this substance is even slightly greater than with fumarate. This supports the view that the system oxaloacetate ⇌ fumarate acts catalytically. Experiments with radioactive carbon [Carson & Ruben, 1940] lead to the same conclusion (see below).

(3) *Adaptation to glycerol.* Figures given in Tables 6 and 7 show that the fermentation of glycerol is relatively slow in washed cells grown on a lactate-containing medium. When the lactate of the culture medium is replaced by glycerol, cells are obtained which ferment glycerol much more rapidly (Table 8). The enzyme systems attacking glycerol are thus partly adaptive.

Table 8. *Adaptation to glycerol*

30 mg. washed cells in 3 ml. 0·05 *M* $NaHCO_3$; gas phase, CO_2.

Culture medium	Substrate added	μl. CO_2 formed 4 hr.	μl. CO_2 formed 20 hr.	μl. succinate formed 20 hr.
Lactate yeast-water	—	41	177	102
,,	0·1 *M* glycerol	115	694	150
Glycerol yeast-water	—	217	631	248
,,	0·1 *M* glycerol	735	1870	732

Effects of oxaloacetate on the fermentation of other substrates

Effects of oxaloacetate similar to that on the fermentation of glycerol were found when mannitol, inositol, arabinose or erythritol were the substrates. The rate of acid formation from these substrates was increased by the addition of oxaloacetate (Table 9). No effects were observed when glucose, lactate or pyruvate were the substrates.

Table 9. *Effect of oxaloacetate on the fermentation of various substrates in* P. Shermanii *(strain of the American Collection of Type Cultures)*

30 mg. cells in 3 ml. 0·05 *M* $NaHCO_3$; CO_2; 5 hr.

Substrates added (final conc.)	µl. acid and CO_2 formed	Extra acid and CO_2 formed from added polyhydric alcohol	
		Without oxaloacetate	With oxaloacetate
None	171	—	—
0·00625 *M* oxaloacetate	375	—	—
Mannitol	291	120	275
Mannitol; 0·0065 *M* oxaloacetate	650		
Inositol	357	186	471
Inositol; 0·00625 *M* oxaloacetate	846		
Arabinose	274	103	171
Arabinose; 0·00625 *M* oxaloacetate	546		
Erythritol	191	20	71
Erythritol; 0·00625 *M* oxaloacetate	446		

Anaerobic metabolism of succinate

Previous investigators [Wood *et al.* 1937] have discussed the formation of propionic acid by decarboxylation of succinic acid and it has been stated that succinate disappears [Wood *et al.* 1937; Fromageot & Bost, 1938] when added together with glucose to suspensions of *P. pentosaceum*. In *P. Shermanii* succinate is not metabolized under anaerobic conditions, either when present alone, or when added together with glucose, glycerol or pyruvate (Table 10). (Aerobically, however, succinate can undergo oxidation.)

Table 10. *Anaerobic metabolism of succinate in* Propionibacterium Shermanii

3 ml. suspension; 27 mg. bacteria; 0·07 *M* $NaHCO_3$; gas phase, CO_2; 370 min.

Substrates added	µl. CO_2 found	µl. succinate found
0·4 ml. 0·1 *M* succinate (=896 µl.)	71	882
0·4 ml. 0·1 *M* glucose	1462	26
0·4 ml. 0·1 *M* glucose; 0·4 ml. 0·1 *M* succinate	1448	942
0·4 ml. *M* glycerol	133	96
0·4 ml. *M* glycerol; 0·2 ml. 0·1 *M* succinate (=448 µl.)	139	536

Alternative mechanisms of succinate synthesis

A second mechanism of succinate synthesis has been suggested by Wood & Werkman [1938; 1940] who assume that a dehydrogenation and condensation of 2 mol. of acetate might lead to succinate:

$$2CH_3.COOH \xrightarrow{-2H} COOH.CH_2.CH_2.COOH. \quad (3)$$

They arrived at this view from the following considerations. The conversion of pyruvate into acetate and CO_2 is the only defined reaction so far known to yield

CO_2 in propionic acid bacteria under anaerobic conditions. If this reaction were the only source of CO_2, acetate and CO_2 should appear in equimolecular quantities. In fact less acetate than CO_2 is formed under certain conditions [Wood & Werkman, 1936, 2]. To explain this Wood & Werkman assumed that some acetate disappears through reaction (3).

This hypothesis is not supported by direct evidence and we do not regard it as satisfactory. In a number of Wood & Werkman's experiments [1936, 2, p. 619, Table II, exps. 1 and 2] the amounts of succinate formed do not make up the deficit in acetate production, even if it is assumed that all the succinate was formed through reaction (3). Thus the hypothesis does not account for the low yield of acetate. However, we accept Wood & Werkman's idea of a further oxidation of acetate, whilst differing from their views on the course of this oxidation. The data on CO_2 production published by Wood & Werkman and by van Niel [1928] suggest that acetate is oxidized to form CO_2. Since succinate cannot be oxidized under anaerobic conditions, it cannot be an intermediate in the oxidation of acetate. The mechanism of acetate oxidation is still completely obscure.

The ability of acetate to undergo oxidation has already been demonstrated by Stone *et al.* [1936] (see also Table 3). The formation of CO_2 from acetate under aerobic conditions is shown in Table 11. Anaerobically addition of acetate

Table 11. *Oxidation and CO_2 formation in the presence of acetate*

P. Shermanii; 10 mg. cells in 3 ml. 0·01 *M* phosphate buffer, *p*H 7·1. Method of Warburg & Yabusoe [1924]; period of incubation 105 min.

Substrate	—	0·03 *M* acetate
μl. O_2 used	135	250
μl. CO_2 (including HCO_3') formed	151	289

Table 12. *Effect of acetate on the fermentation of oxaloacetate*

30 mg. cells, *P. Shermanii*, in 3 ml. 0·04 *M* $NaHCO_3$; 5 % CO_2 in N_2; period of incubation 21 hr. The bacteria were shaken before the experiment for 18 hr. in the presence of O_2 to remove preformed intracellular substrates.

Added substrates	0·5 ml. 0·1 *M* oxaloacetate	0·5 ml. 0·1 *M* oxaloacetate, 0·5 ml. 0·5 *M* acetate
μl. succinate formed	352	446

causes an increase of the yield of succinate from oxaloacetate (Table 12). We take this as an indication of a reduction of oxaloacetate at the expense of an oxidation of acetate. The hypothesis of the oxidation of acetate also explains the high values for the ratio propionate/acetate which have often been found. According to the original concept of propionic acid fermentation [Fitz, 1878] 3 triose equivalents are assumed to form 2 mol. of propionic acid and 1 mol. of acetic acid and of CO_2. These quantities have been found in many experiments but there are also many cases where the yield of acetate is low. Wood & Werkman [1936, 2] found the ratio propionate/acetate to be between 2·13 and 14·72. These deviations from the expected value 2 are explained by the assumption that acetate undergoes decomposition to CO_2. The reduction equivalent for this oxidation may be the formation of either propionate or of succinate.

Experiments with other strains of propionic acid bacteria

Further experiments were undertaken to see whether the reduction of oxaloacetate and the catalytic effects of oxaloacetate on the fermentation of glycerol

Table 13. *Fermentation of oxaloacetate and glycerol in various strains of propionic acid bacteria*

30°; 0·045 *M* $NaHCO_3$; gas phase CO_2; the data refer to 3 ml. suspension.

Strain	mg. cells in 3 ml. suspension	Substrates added (final conc.)	Period of incubation min.	μl. CO_2 formed (free CO_2 from substrate plus CO_2 from bicarbonate)	μl. succinate formed
P. Freudenreichii	28	—	120	56	—
	28	0·0133 *M* oxaloacetate	120	215	220
	28	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	120	675	251
	28	0·1 *M* glycerol	120	120	77
P. Jensenii	40	—	160	102	59
	40	0·0133 *M* oxaloacetate	160	561	571
	40	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	160	1380	846
	40	0·1 *M* glycerol	160	301	164
P. Shermanii	30	—	240	147	33
	30	0·0133 *M* oxaloacetate	240	458	440
	30	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	1960	1150
	30	0·1 *M* glycerol	240	227	61
P. Thönii	27	—	240	155	—
	27	0·0133 *M* oxaloacetate	240	257	49
	27	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	970	96
	27	0·1 *M* glycerol	240	407	76
P. rubrum	27	—	240	116	—
	27	0·0133 *M* oxaloacetate	240	319	25
	27	0·0133 *M* oxaloacetate, 0·1 *M* glycerol	240	1025	81
	27	0·1 *M* glycerol	240	312	87

occur generally in propionic acid bacteria. Five strains of the American Collection of Type Cultures were tested and on the whole the results (Table 13) are similar to those obtained with *P. Shermanii*. In each case oxaloacetate is reduced to succinate, and glycerol increases the rate of succinate formation. The rate of acid production from glycerol was also increased by the addition of oxaloacetate. In the case of *P. Thönii* and *P. rubrum* the yield of succinate was low, and a further investigation of this difference showed that fumarate and malate appear instead of succinate in these strains (Table 14).

Table 14. *Fermentation of oxaloacetate and glycerol in* P. Thönii

39 mg. cells in 3 ml. 0·05 *M* $NaHCO_3$; CO_2; 5 hr. For the calculation of fumarate + *l*(−)-malate it was assumed that the ratio $\frac{l(-)\text{-malate}}{\text{fumarate}} = 3{\cdot}54$ for temp. 30°.

Substrates added	0·027 *M* oxaloacetate	0·027 *M* oxaloacetate 0·1 *M* glycerol	0·1 *M* glycerol
Total CO_2 evolved (μl.)	629	2360	700
Succinate formed (μl.)	45	78	119
Fumarate formed (μl.)	78	141	89
Fumarate + *l*(−)-malate (calc.)	354	640	404

Discussion

The evidence in support of scheme (2). The most important part of the evidence in favour of scheme (2) is the simultaneous formation of fumarate and succinate from glycerol (Tables 5 and 14). Since the reaction succinate → fumarate does not occur under anaerobic conditions (Table 10), fumarate formed anaerobically cannot have arisen from succinate: if the formation of succinate by reduction is rejected, the improbable assumption of two separate mechanisms for the formation of succinate and of fumarate must be made.

The fact that all the reactions postulated by (2) can occur, lends much further support to the scheme, for if an added substance is readily metabolized, it is very likely to be a normal metabolite.

Biological significance of the synthesis of oxaloacetate. As shown by Wood & Werkman [1938] glycerol is fermented in propionic acid bacteria in two ways:

$$CH_2OH.CHOH.CH_2OH \longrightarrow CH_3.CH_2.COOH + H_2O,$$
$$CH_2OH.CHOH.CH_2OH + CO_2 \longrightarrow COOH.CH_2.CH_2.COOH + H_2O.$$

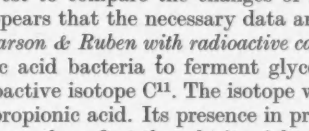
The significance of the second form of glycerol fermentation lies probably in the fact that it produces the oxaloacetate and fumarate required as hydrogen carriers.

It would be of interest to compare the changes of free energy in the two fermentations, but it appears that the necessary data are not yet available.

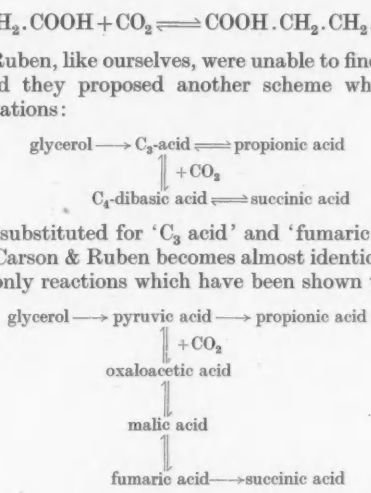
The experiments of Carson & Ruben with radioactive carbon. Carson & Ruben [1940] allowed propionic acid bacteria to ferment glycerol in the presence of CO_2 containing the radioactive isotope C^{11}. The isotope was found to be present in succinic acid and in propionic acid. Its presence in propionic acid was somewhat unexpected and the authors first thought it might be due to the following reversible reaction:

$$CH_3.CH_2.COOH + CO_2 \rightleftharpoons COOH.CH_2.CH_2.COOH.$$

However Carson & Ruben, like ourselves, were unable to find evidence in support of this reaction and they proposed another scheme which explains all the experimental observations:



If 'pyruvic acid' is substituted for 'C_3 acid' and 'fumaric acid' for 'C_4-dibasic acid' the scheme of Carson & Ruben becomes almost identical with the following scheme comprising only reactions which have been shown to occur:



This scheme accounts for all the facts so far observed. It should be noted that the presence of radioactive carbon in propionic acid is explained if the C_4-

dibasic acid is fumaric acid, but it is not explained if it is malic acid. For malic acid, formed by reaction (2), would on breakdown yield a pyruvic acid containing no isotope. On the other hand, fumaric acid containing an active carbon atom in the carboxyl group would be converted into two different malic acids, viz. *COOH.CHOH.CH_2.COOH and COOH.CHOH.CH_2.*COOH (the asterisk denotes the isotope). On breakdown the first would yield a pyruvic acid (and on reduction of the latter a propionic acid) containing the isotope, whilst the second would lose the isotope.

The appearance of the isotope in the propionic acid formed from glycerol may be taken as further evidence in support of the view that the system oxaloacetate $\rightleftharpoons$ fumarate acts as a hydrogen carrier in the fermentation of glycerol. It is direct proof of the reversibility, under 'normal' conditions, of the reactions of the scheme (2) leading from pyruvate and CO_2 to fumarate.

Summary

1. Propionic acid bacteria readily reduce oxaloacetate to succinate. Six different strains were examined. Fumarate and *l*(−)-malate are formed as intermediate metabolites. The hydrogen required for the reduction of oxaloacetate can be supplied by several reactions, e.g. the conversion of pyruvate into acetate.

2. Fumarate and *l*(−)-malate are readily reduced to succinate in *P. Shermanii*.

3. A powerful fumarase is present in *P. Shermanii*.

4. The rates of succinate formation from oxaloacetate, fumarate and malate are sufficiently high to allow the assumption that these acids are intermediates in the formation of succinate from glycerol, in accordance with scheme (2).

5. Fumarate is shown to be formed when glycerol is anaerobically fermented by *P. Shermanii*. This is considered as evidence in support of (2).

6. Fumarate, malate or oxaloacetate catalytically accelerate the fermentation of glycerol. It is concluded that these acids act as intermediary hydrogen carriers in the fermentation of glycerol. It is pointed out that the experiments of Carson & Ruben with radioactive carbon support this view.

7. Similar catalytic effects of oxaloacetate were observed when mannitol, inositol, arabinose or erythritol were the substrates of fermentation.

8. The view that succinate is synthesized in propionic acid bacteria from 2 mol. of acetate is rejected. Acetate can be oxidized aerobically, and probably anaerobically, to form CO_2. The oxidative conversion of acetate into CO_2 explains the low yields of acetate observed by previous investigators.

9. The facts concerning the synthesis of succinate in propionic acid bacteria are all explained by scheme (2), according to which the synthesis of oxaloacetate from pyruvate and CO_2 is the primary step in the synthesis of the 4 carbon chain.

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72. CYTOCHROME SYSTEM IN *BACTERIUM COLI COMMUNE*

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The aerobic respiration of *Bact. coli*, like that of *Bacillus subtilis*, baker's yeast and other aerobic cells, is strongly and reversibly inhibited by KCN, H_2S, NaN_3 and CO, but, unlike that of *B. subtilis* or cells of baker's yeast, the inhibition with CO is not light-sensitive.

The absorption spectrum of cytochrome in *B. coli* differs markedly from that of *B. subtilis* or yeast cells. Thus, the band *a* is absent from *B. coli*, and is replaced by a hardly perceptible shading a_1 at 590 mμ and a feeble band a_2 at 628 mμ which is similar to the band a_2 of *Azotobacter*. This band in organisms exposed to CO moves to 634 mμ, on oxidation it is shifted to 645 mμ and in presence of KCN and O_2 it disappears completely[1] [Keilin, 1934; Fugita & Kodama, 1934]. The band a_2 belongs therefore to an autoxidizable haemochromogen compound which reacts with KCN and with CO. According to Lemberg & Wyndham [1937] this compound can be considered as a bile pigment haemochromogen with an open tetrapyrrolic chain kept together in a ring form by the central iron atom. Lemberg ascribes to a_2 the structure of a biliviolin-haemochromogen which is obtained by dehydrogenation of verdohaemochromogen. The component a_2 appears therefore to belong to a bile pigment derivative of haematin *a*, and, as in all the derivatives with an open tetrapyrrolic chain [Holden & Lemberg, 1939], its absorption spectrum is devoid of the Soret or γ-band.

The properties of a_2, namely its reactions with O_2, CO and KCN, suggest that in *B. coli*, as in *Azotobacter* [Negelein & Gärischer, 1934], this component may act as cytochrome oxidase. On the other hand, the failure to find any parallelism between the concentration of a_2 and the respiratory activity of cells containing this component leaves open the possibility of the existence in *B. coli* of yet another autoxidizable haematin compound acting as cytochrome oxidase and capable of oxidizing the component b_1.

The bands *b* and *c* of a typical cytochrome are replaced in *B. coli* by one band which lies at about 560 mμ and which represents the α-band of a non-autoxidizable haemochromogen compound b_1. This band is asymmetric and shows on its short-wave side a reinforcement which, according to Yamagutchi [1937], may represent the band *c* masked by an extension of the band b_1.

On freezing and cooling the suspension of *B. coli* to the temperature of liquid air, the short-wave side of the band b_1 becomes greatly intensified and appears at 551 mμ united by a shading to a much fainter band at 559 mμ. The α-band of cytochrome *c* which, under similar conditions, is also markedly intensified is, however, shifted from its usual position at 549·5 to 547 mμ. This clearly shows, even if we assume that the asymmetric band b_1 of *B. coli* is composed of two

[1] In some other strains of *B. coli* and in *Proteus vulgaris* Yamagutchi [1937] failed, however, to detect any reaction between a_2 and KCN.

fused bands belonging to two distinct compounds, that neither of them can be identified with the true components *b* and *c* of cytochrome.

The component b_1 in living intact cells of *B. coli* does not react with KCN, NaN_3 or CO, which however inhibit its oxidation.

Catalytic activity of cytochrome in Bact. coli

Unlike *B. subtilis* and many other aerobic cells, *B. coli*, even when free from metabolites, is incapable of oxidizing *p*-phenylenediamine or 'Nadi' reagent [Happold, 1930]. This may be partly explained by the absence from *B. coli* of cytochrome *c* which, as was previously shown [Keilin & Hartree, 1938], is essential for these catalytic oxidations. A washed suspension of *B. coli* can neither reduce nor oxidize added cytochrome *c*, which is not surprising considering that cytochrome *c*, being a comparatively large molecule, cannot diffuse through the cell wall of *B. coli* and thus come in contact with its intracellular oxidizing systems. The direct reaction between cytochrome *c* and the oxidizing systems of *B. coli* can only be determined when crushed micro-organisms, in which these systems are exposed, are brought into contact with a solution of cytochrome *c*.

The study of this inter-reaction was carried out in a series of spectroscopic and manometric experiments. For this purpose *B. coli* were grown in the usual way on a peptone-agar medium[1] in about thirty large Roux flasks. The organisms after 24 hr. growth were collected, washed several times in saline solution and mixed with 30 ml. of phosphate buffer solution at *p*H 7 to a thick suspension which was ground for about 1 hr. in a Booth & Green [1938] bacterial mill. The ground preparation thus obtained gave on centrifuging three distinct layers: the top layer (*A*) of opalescent fluid, the intermediate or granular layer (*B*) composed of crushed bacteria and the bottom layer (*C*) of intact bacteria. The first two layers (*A* and *B*), collected separately and dialysed for at least 14 hr. against a large amount of 0·02 *M* phosphate buffer solution (*p*H 7·3), were found to possess similar properties, except that the fraction *B* displayed a stronger absorption spectrum and a greater catalytic activity than fraction *A*. The fraction *B*, which was used for most of the observations recorded below, did not take up O_2 and its cytochrome remained in a completely oxidized state. If, however, substrates such as succinic, lactic or formic acids were added to this preparation, they became rapidly oxidized and the preparation took up O_2 vigorously. Cytochrome in such preparations shaken with air undergoes rapid oxidation and reduction.

As all the components of the oxidizing systems in this preparation are now exposed they can easily be brought into contact with cytochrome *c*.

Spectroscopic experiments

For spectroscopic study, a small amount of pure cytochrome *c* prepared from heart muscle as previously described [Keilin & Hartree, 1937] was added to fractions *A* or *B* of crushed bacteria in presence and in absence of substrate. Spectroscopic examination of these mixtures showed that the dialysed fractions (*A* or *B*) of crushed *B. coli*, unlike similar preparations of baker's yeast, do not oxidize reduced cytochrome *c* even when the mixtures are vigorously shaken with air. The same preparations in the absence of substrate do not reduce oxidized *c*. They reduce this compound, however, in the presence of substrates

[1] The nutrient medium is composed of 20 g. peptone, 20 g. 'Lab. Lemco,' 10 g. NaCl, 60 g. agar and 2 l. water.

such as succinic or lactic acids. Once reduced, cytochrome *c* does not undergo reoxidation on shaking the mixture with air, while components a_2 and b_1 of cytochrome proper to *B. coli* undergo normal and rapid reoxidation.

Manometric experiments

It has been shown [Keilin, 1930; Keilin & Hartree, 1938] that the addition of cytochrome *c* to enzyme preparations obtained from heart muscle, liver or kidney, greatly increases the rates of oxidation of different substances, such as cysteine, succinic acid, *p*-phenylenediamine or adrenaline, catalysed by these preparations. The addition of cytochrome *c* increases also the rates of oxidation of lactic acid or of *p*-phenylenediamine by enzyme preparations obtained from crushed cells of baker's yeast.

Similar experiments were carried out in Barcroft differential manometers at 39° with both fractions (*A* and *B*) of the preparation obtained from crushed *B. coli*. The enzyme preparations were suspended in 3 ml. *M*/15 phosphate buffer, *p*H 7·3. The substrates, such as succinic or lactic acids, or *p*-phenylenediamine, were added from dangling tubes which were dislodged only after the temperature of the manometers had been equilibrated and the taps closed. The final concentrations of substrate were *M*/50. The clear fraction (*A*) was used in these experiments in a higher concentration than the granular and more active fraction (*B*). In some experiments the enzyme preparation received 0·2 ml. of 1 % cytochrome *c*, or 10^{-3} *M* methylene blue and cyanide.

Table 1

B. coli enzyme prep. ml.	Cyto-chrome *c*	*p*-pheny-lenedia-mine	Succinic acid	Lactic acid	KCN	M.B.	O_2 uptake μl. in 25 min.
0·5 (*A*)	−	+	−	−	−	−	6
+	+	+	−	−	−	−	6
+	−	−	+	−	−	−	174
+	+	−	+	−	−	−	160
+	−	−	+	−	+	−	17
+	−	−	+	−	+	+	66
+	−	−	−	+	−	−	215
+	+	−	−	+	−	−	212
0·1 (*B*)	−	+	−	−	−	−	13
+	+	+	−	−	−	−	16
+	−	−	+	−	−	−	198
+	+	−	+	−	−	−	160
+	−	−	−	+	−	−	166
+	+	−	−	+	−	−	164
+	−	−	−	+	+	−	11
+	−	−	−	+	+	+	174

The results of these experiments, which are summarized in Table 1, show that the oxidation of succinic or lactic acid which is actively catalysed by preparations of crushed *B. coli* is not accelerated by the addition of cytochrome *c*. Under similar conditions cytochrome *c* greatly increases the rate of catalytic oxidation of these substances by crushed cells of baker's yeast. Moreover, crushed *B. coli*, even on addition of *c*, oxidized neither *p*-phenylenediamine nor other artificial chromogens such as adrenaline, Nadi reagent, quinol or catechol. The main conclusion which can be drawn from these experiments is that cells of *B. coli* contain only the systems which are capable of reducing cytochrome *c* but are completely devoid of a catalyst capable of oxidizing the reduced *c*. Such a catalyst is, however, present in cells containing the typical cytochrome system. It was shown recently [Keilin & Hartree, 1939] that these cells, in addition to

components *a*, *b* and *c* of cytochrome, contain also a fourth component described as a_3. This component is thermolabile and autoxidizable; it forms reversible compounds with KCN, H_2S, NaN_3 and CO and the absorption bands of its CO compound lie at about 591 and 430 mμ.

It was also shown that the catalytic activities of a tissue preparation measured by the velocity of oxidation of *p*-phenylenediamine in presence of an excess of *c* appear to depend on the concentration of the component a_3. This component seems, therefore, to possess all the properties of cytochrome oxidase and directly or indirectly must be responsible for the catalytic oxidation of cytochrome *c*. It is now conceivable that the failure of *B. coli* to oxidize cytochrome *c* is due to the absence from these micro-organisms of the component a_3 which invariably accompanies the cytochrome *a*.

The results obtained for *B. coli* will probably apply to cells of some other micro-organisms (bacteria and yeast) as well as some cells of higher organisms, in which the cytochrome system is devoid of components *a*, a_3 and *c*.

The fact that cytochrome *c* cannot act as a carrier between the dehydrogenases of *B. coli* and their cytochrome oxidase does not exclude the possibility that it may act as a link uniting into an artificial system the dehydrogenases of *B. coli* with cytochrome oxidase of heart muscle. To test such a possibility, the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed bacteria were determined in presence and absence of cytochrome *c* and of 0·1 ml. of a fresh heart-muscle preparation, which can rapidly oxidize the reduced *c*. The results of these experiments, summarized in Table 2, clearly show

Table 2

B. coli enzyme 0·05 ml. (*B*)	Cytochrome *c*	Cytochrome oxidase 0·1 ml.	Lactate	Formate	O_2 uptake μl.
+	−	−	+	−	136
+	+	−	+	−	156
+	+	+	+	−	174
+	+	+	−	−	50
+	−	−	−	+	140
+	+	+	−	+	123
+	−	−	+	−	168
+	+	+	+	−	138
+	+	+	+	+	285

that the rates of oxidation of lactic and formic acids by the enzyme preparation obtained from crushed *B. coli* are not affected by the addition of cytochrome *c* and cytochrome oxidase prepared from heart muscle. The substrate molecules, activated by their specific dehydrogenases, appear to react with molecular O_2 only through the cytochrome system proper to *B. coli* and not through the cytochrome system of heart muscle added to the *B. coli* preparation. These results suggest that the components of each of the insoluble dehydrogenase systems, together with those of cytochrome, are intimately bound to the protein of a single colloidal particle, forming thus a unit of a complete oxidizing system. The efficiency of such a system is naturally assured by the mutual accessibility of its components, which depends on their spatial distribution and orientation. This view explains the different degree of inhibition by CO of the oxidation by resting *B. coli* of succinic, lactic and formic acids [Cook *et al.* 1931] without postulating the existence of three different cytochrome oxidases. In fact the three different values of *K* (partition constant of oxidase between O_2 and CO) obtained by these workers in their experiments with the oxidation of these

three substances may simply be due to the different degree of accessibility between each of the dehydrogenases and the components of cytochrome bound in the same colloidal particle. The efficiency of the oxidizing systems of the cell and the co-ordination of the multiple reactions simultaneously proceeding within the cell must therefore depend not only on the integrity of all the components of these systems but also on that of the colloidal structure of the cell which supports them and regulates their mutual accessibility.

Summary and conclusions

1. Contrary to the view of Yamagutchi, *Bact. coli* are completely devoid of cytochrome *c*.

2. The preparations obtained from crushed *B. coli* in which all the oxidizing systems are exposed are not capable of oxidizing reduced cytochrome *c* added to them. *B. coli* and probably some other cells are completely devoid of systems or catalysts capable of oxidizing cytochrome *c*.

3. It is therefore not surprising that the preparations of crushed *B. coli*, even on addition of cytochrome *c*, oxidize neither *p*-phenylenediamine nor other chromogens and that the oxidation of succinic and lactic acids by these preparations is not accelerated by the addition of *c*.

4. The failure by *B. coli* to oxidize cytochrome *c* is due to the absence from the cells of this micro-organism of the component a_3 which invariably accompanies cytochrome *a* and which directly or indirectly is responsible for the oxidation of cytochrome *c*.

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73. VITAMIN A STUDIES WITH RATS AND PIGS

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Many aspects of the metabolism of vitamin A and of its precursors are still insufficiently known; this is especially true with regard to information available for animals other than the rat. Two of us (A. S. F. and S. K. K.) have for some time been interested in the importance of vitamin A in pig feeding. For reasons both practical and scientific we wanted to gain more knowledge of the utilization by the pig of certain carotenoids, of the relative value for the pig of carotene and of preformed vitamin A and of the minimum vitamin A requirements of this animal. Another of the authors (T. H. M.) has studied on rats the biological activity of vitamin A alcohol and of natural vitamin A esters and further comparisons using a different animal were thought likely to be of value. The present joint paper gives the results of all these inquiries. The biological tests were carried out at Shinfield and the chemical and physical manipulations were mostly done in the laboratories of The British Drug Houses, Ltd.

It is generally accepted that in order to exhibit vitamin A activity a carotenoid pigment must contain a β-ionone ring or rings. Recently, however, Lund [1938] and Petersen [1939] reported that zeaxanthin was effective in preventing symptoms of vitamin A deficiency in pigs, but that 'xanthophyll' (lutein) was inactive in this respect. If confirmed, this finding would be of considerable importance, not only because it would demonstrate the unique ability of the pig to convert a dihydroxycarotene into vitamin A, but also from the practical standpoint of pig feeding. Maize, an important pig food, contains relatively more zeaxanthin than carotene and cryptoxanthin [Kuhn & Grundman, 1934]. The observation of Lund [1938] that the pig can make use of dihydroxy-β-carotene but not of dihydroxy-α-carotene (lutein, xanthophyll) is certainly puzzling, as it implies that a hydroxy-β-ionone ring is utilized in one case but not in the other. The preparation of zeaxanthin used by Lund [1938] was admittedly contaminated with carotene, and we felt that for this and other reasons a reinvestigation of the value of zeaxanthin as a source of vitamin A for pigs was amply justified.

Pure β-carotene has, by definition, a potency of $1{\cdot}67 \times 10^6$ I.U./g., while, according to most recent data [Holmes & Corbett, 1937; Mead *et al.* 1939; Baxter & Robeson, 1940], the potency of pure vitamin A measured on the rat is nearly double this figure, i.e. about $3{\cdot}0$–$3{\cdot}3 \times 10^6$ I.U./g. These figures indicate that this animal, even under most favourable conditions, can form only one molecule of vitamin A from one molecule of β-carotene. This is what one would expect on theoretical grounds, as there is no reason to suppose that the fission of the β-carotene molecule takes place symmetrically [Morton, 1940].

The information regarding other animals is less definite, but Guilbert *et al.* [1940] have shown that cattle, sheep, pigs and horses are, if anything, less efficient than rats in converting carotene into vitamin A. Full references to

this work will be found in a recent paper by Hart [1940]. It was found that to cover minimum requirements the four species needed 5·3–7·1 μg. of vitamin A[1] per kg. body weight but that 24–34 μg. of carotene, either from artificially dried lucerne or as a preparation (88 % β- and 12 % α-carotene) in oil, were required for the same purpose. The animals obtained therefore a yield of only about half a molecule of vitamin A from one molecule of β-carotene. At least 5–10 times the minimum level is recommended in practice by the American authors. In this country, Dunlop [1934] found that 14 mg. of carotene per 100 lb. of food are insufficient for normal growth of pigs, while 62 mg./100 lb. give normal growth with storage of the vitamin. These values correspond for a 50 kg. pig to 12 and 53 μg. respectively of carotene per kg. body weight [Dunlop, 1933] and agree with the American experience. According to Lund [1938] the minimum requirement of pigs up to 40 kg. in weight does not exceed 30 μg./kg. body weight.

The relation between the biological activity of materials containing vitamin A and their spectral absorption at 328 mμ remains uncertain in spite of the numerous investigations that have been devoted to this subject [e.g. Coward *et al.* 1931; 1932; Morgan *et al.* 1935; Hume, 1937; 1939; Moll & Reid, 1939]. Values ranging from below 1000 to over 3000 have been found for the factor for converting $E^{1\%}_{1\,cm.}$ 328 mμ to biological units, and statistical analyses have in some cases [Hume, 1939; Morgan *et al.* 1935] indicated that the differences between the factors assigned to different materials are greater than the variations to be expected on account of the errors inherent in biological tests.

Although a survey of the available results reveals no hard and fast relation between the type of material tested and its appropriate conversion factor, certain generalizations hold with few exceptions. Some commercial vitamin A concentrates prepared from fish liver oils by saponification tend to give low factors, as also do mammalian liver oils and the corresponding concentrates. Higher values are usually given by unhydrolysed fish liver oils and in particular by fresh medicinal cod-liver oils and by the reference oil of the United States Pharmacopoeia. Exceptionally high factors have been claimed by Moll & Reid [1939] for samples of a commercial vitamin A concentrate which is prepared without saponification.

A number of explanations for the wide variation in experimentally determined conversion factors have been suggested. Pritchard *et al.* [1937] have, for example, shown that mammalian liver oils contain a factor which contributes to spectral absorption at 328 mμ but has little biological activity. The presence of this material probably accounts for the low conversion factors found for mammalian liver oils. The low biological activity of some fish liver oils relative to their extinction coefficient at 328 mμ may similarly be due to the presence of oxidized vitamin A [Robinson, 1938], cyclized vitamin A [Edisbury *et al.* 1932; Embree, 1939], vitamin A_2, or possibly other substances having little or no biological activity. Another explanation is that different oils may contain different proportions of the many possible isomers of vitamin A.

Concentrates prepared by saponification have most frequently been found to give the lowest conversion factors and it is therefore conceivable that this operation affects the potency of the vitamin. Since the vitamin A in fish liver oils is esterified, while that in concentrates prepared by saponification is free

[1] Wherever quantities of vitamin A are in this paper expressed in terms of weight it has been assumed that pure vitamin A has $E^{1\%}_{1\,cm.}$ 328 mμ = 1800 [Mead *et al.* 1939; Baxter & Robeson, 1940]. Quantities quoted from the literature have been recalculated to this basis to facilitate comparison.

[Bacharach & Smith, 1928; Reti, 1935; Hamano, 1935; Hickman, 1937; Tischer, 1938], the question arises as to whether a lower conversion factor should be ascribed to vitamin A alcohol than to vitamin A esters.

The existing evidence consists for the most part in experiments in which crude fish oils and crude concentrates were assayed against β-carotene on rats. In 1936 careful assays of a sample of halibut liver oil and of its non-saponifiable fraction were carried out in ten laboratories. Although the results [Hume, 1937; 1939] showed a higher conversion factor for the oil than for the concentrate, the latter was found by the spectrographic test to have deteriorated during the test period by an amount sufficient to account for the discrepancy. Emmet & Bird [1937] reported that tests carried out on a fish liver oil and on concentrates prepared from it by varying degrees of saponification indicated that a lowering of conversion factor occurred which ran parallel with the progress of hydrolysis. Hickman [1939] stated that distilled esters of vitamin A had the same conversion factor as the vitamin A in crude oils, whereas concentrates prepared by saponification always had a smaller factor which might be less than half that of the oils. Moll & Reid [1939], using a bioassay technique differing greatly from that normally used in England, found conversion factors of from 3500 to 3700 for a commercial vitamin A concentrate containing vitamin A esters. To the non-saponifiable fraction of this concentrate they assigned a factor of 1800.

The problem under discussion is complicated by the following considerations: (*a*) vitamin A alcohol in concentrates is more readily oxidized than vitamin A esters present in fish liver oils; (*b*) oxidation products of vitamin A absorb strongly in the neighbourhood of 328 mμ but have little or no biological activity [Edisbury *et al.* 1932; Robinson, 1938]. It is therefore to be expected that low conversion factors will be found for commercial vitamin A concentrates which may have become partially oxidized during preparation and storage, as well as for concentrates which have been carefully prepared in the laboratory but which have not been fully protected against oxidation during the bioassay period. It is evident that the conversion factor applicable to any material containing vitamin A depends in the first place on its content of impurities affecting the spectrographic test. It follows that evidence of the true relation between the biological activities of free and esterified vitamin A is obtainable only from tests on materials free from such impurities.

One object of the present experiment was to discover whether any difference could be detected between the potencies of vitamin A alcohol and natural vitamin A esters when both had been freed as completely as possible from spectrographic impurities by molecular distillation. Simultaneous tests of the purified materials were carried out on pigs and on rats. A more comprehensive comparison of similarly purified materials was carried out on rats by Dr Underhill at The British Drug Houses, Ltd., before the experiment on pigs was undertaken. His results will form the subject of a separate paper.

Experimental

Preparation of materials

Vitamin A alcohol and purified natural vitamin A esters. Our principal object in the purification of these materials was to eliminate as completely as possible substances such as oxidized and cyclized vitamin A which give rise to irrelevant absorption at 328 mμ.

Vitamin A alcohol having $E^{1\%}_{1\,cm.}$ 328 mμ = 1600 was used for these experiments. It was prepared as described by Mead *et al.* [1939] by molecular distillation of the non-saponifiable fraction from a rich fish liver oil.

The natural vitamin A esters were made by purifying an ester concentrate obtained by distilling unhydrolysed fish liver oil. The crude material, a dark brown semi-solid (Carr-Price value 21,000), was dissolved in light petroleum and the solution was filtered through a column of activated alumina. The column was rinsed with the solvent until the brown zone, which at first occupied the top half, reached nearly to the bottom. The filtrate and rinsings were concentrated under diminished pressure, leaving a pale yellow oil which was distilled in a cyclic molecular still of the Hickman type. A first fraction consisting of a small quantity of red oil containing some cyclized vitamin A and vitamin A alcohol was obtained by circulating the distilland over the distilling surface successively at 80°, 90°, 100°, 120°, 130°, 140°, 150° and 155°. A second fraction consisting of vitamin A esters contaminated with sterols and traces of vitamin A alcohol was removed by cycles at 160°, 165° and 170°. The third fraction, which was used to prepare the feeding solutions for this experiment, was collected by circulation once at 175° and twice at 180°. It consisted of a pale yellow oil having Carr-Price value 33,000 and $E^{1\%}_{1\,cm.}$ 328 mμ = 660.

If pure vitamin A alcohol has an extinction coefficient $E^{1\%}_{1\,cm.}$ 328 mμ = 1800, this concentrate of the natural esters contained 36·7 % of vitamin A. On the assumption that the average molecular weight of the fatty acids combined with the vitamin A is of the same order as that of oleic acid, the vitamin A ester concentrate contained about 73 % of natural vitamin A esters. The remainder probably consisted chiefly of fatty triglycerides and fatty acid esters of cholesterol.

β-Carotene. A specimen of commercial crystalline mixed carotenes was purified by chromatographic adsorption on magnesia followed by crystallization from benzene-ethanol. At no time during the preparation was the substance exposed to a temperature exceeding 45°. The product had M.P. 184° (corr.) and $E^{1\%}_{1\,cm.}$ 456 mμ = 2400 (in cyclohexane).

Zeaxanthin was obtained by hydrolysis of the naturally occurring dipalmitate (physalien). The hydrolysate was carefully separated by chromatography from carotene, cryptoxanthin and other carotenoids present in crude extracts of *Physalis*. The seed pods from freshly picked *Physalis* (approx. 30 kg.) were separated from the berries, covered immediately with alcohol and set aside for a month. The alcohol was run off and the pods were air-dried, first at room temperature and finally overnight at 37°, before being pulverized in a ball-mill from which the air had been displaced by CO_2. The powder was immediately extracted with successive portions of light petroleum until all the pigment had been removed. The combined extracts were evaporated in an atmosphere of N_2 under diminished pressure to a small bulk; sufficient benzene was added to give a clear solution (vol. approximately 1·5 l.), which was treated with alcohol (4·5 l.). After a short time, the crude physalien was collected, washed with alcohol, and immediately recrystallized by the addition of warm alcohol to its solution in warm benzene. Yield 57 g. The physalien was hydrolysed and the crude zeaxanthin purified in several small batches as follows.

Physalien (12·5 g.) was dissolved in benzene (125 ml.). The solution was diluted with light petroleum (1875 ml.) and shaken for 8 hr. at room temperature with a solution of KOH (150 g.) in methyl alcohol (625 ml.). On the addition of distilled water (about 1 l.) the zeaxanthin separated at the interface between the petroleum and aqueous-methanol phases. The upper and lower layers were

siphoned off leaving the zeaxanthin suspended in a small volume of liquid from which it was separated by filtration. The product was thoroughly washed on the filter with 50% methyl alcohol and dried in a desiccator. Yield 6·5 g. from 12·5 g. physalien. For purification the crude material was dissolved in warm ethylene dichloride (3·5 l.) and the solution, after being cooled to room temperature, was filtered through a column (ca. 11 × 55 cm.) containing 3 kg. of 'heavy' magnesia. The chromatogram was developed with ethylene dichloride (2·5 l.) followed by ethylene dichloride mixed with 15% benzene (1 l.) until the intermediate orange band containing the zeaxanthin was well separated from the zones above and below it. The appropriate zone was dissected out and eluted with chloroform containing methyl alcohol (5%). The eluate was washed several times with water in order to remove methyl alcohol, dried (Na_2SO_4) and evaporated under diminished pressure to a small volume from which the zeaxanthin crystallized on the addition of light petroleum. After recrystallization from chloroform (150 ml.) the product melted at 214° (uncorr.). Yield 3·35 g. A solution in ethanol showed absorption bands at 454 and 480 mμ:

$$E^{1\%}_{1\,cm.}\ 454\ m\mu = 2520. \quad E^{1\%}_{1\,cm.}\ 480\ m\mu = 2230.$$

Strain [1938] gives the following data for pure zeaxanthin (M.P. 212–213°). Absorption bands (in ethanol) at 423·5 (weak), 451·5 and 483 mμ. The following extinction coefficients are taken from the curve on p. 102 of Strain's monograph:

$$E^{1\%}_{1\,cm.}\ 454\ m\mu = 2470. \quad E^{1\%}_{1\,cm.}\ 480\ m\mu = 2190.$$

Preparation and storage of the animal feeding solutions

Solvent oil. Arachis oil specially selected on account of low peroxide content and the negligible absorption given at 328 mμ was used for preparing the test solutions after treatment with quinol (0·01%).

Solutions of vitamin A alcohol. In calculating the quantities to be used for making the solutions of vitamin A alcohol and vitamin A esters, it was assumed that the potency in I.U./g. is equal to the extinction coefficient multiplied by 1600. A solution containing approximately 500 I.U./g. for feeding to the pigs was prepared by dissolving 1·82 g. of vitamin A alcohol ($E^{1\%}_{1\,cm.}$ 328 mμ = 1600) in 541·1 g. of arachis oil and diluting part of this solution (273·7 g.) to 4·87 kg. A solution containing 200 I.U./g. for the rat experiment was prepared from this by appropriate dilution with arachis oil. The extinction coefficients of the two solutions were measured immediately after preparation and again at intervals during the biological experiments. The results of these tests and of those on the solutions of vitamin A esters, β-carotene and zeaxanthin are given in Table 1.

Solutions of vitamin A esters. A solution containing 500 I.U./g. of the purified vitamin A esters in arachis oil was prepared by dilution in two stages in a manner similar to that used for making the vitamin A alcohol solution. From this the 200 I.U./g. solution for the rat experiment was prepared by dilution with the same solvent.

Solutions of β-carotene. The biological activity of the carotene was assumed to be 1,670,000 I.U./g. The following procedure was adopted in order to avoid the risk of decomposing the carotene by exposure to temperatures above 40°. β-Carotene (1·314 g.) was dissolved in pure benzene (300 ml.) and approximately one-third of this solution was added to about 1 l. of arachis oil contained in a 5 l. flask. The greater part of the benzene was removed in a vacuum and the solution transferred to a tared 10 l. flask. These operations were repeated twice, using the remaining two-thirds of the solution of carotene in benzene, the last

Table 1. *Spectrophotometric measurements on the feeding solutions*

Substance	Correct potency I.U./g.	Date	$E^{1\%}_{1\,cm.}$	I.U./g.
Vitamin A alcohol	500	7. ii. 40	0·303	485
			0·304	
		13. iii. 40	0·276	454
			0·292	
		1. v. 40	0·286	458
		1. viii. 40	0·295	465
			0·286	
	200	8. ii. 40	0·121	194
		12. iii. 40	0·115	185
Vitamin A esters	500	6. i. 40	0·300	488
			0·310	
		13. iii. 40	0·300	480
		1. v. 40	0·297	475
		1. viii. 40	0·271	435
			0·271	
		3. viii. 40*	0·295	473
	200	6. i. 40	0·121	194
		12. iii. 40	0·118	189
β-Carotene	500	3. i. 40	0·710	494
		13. iii. 40	0·750	522
		1. v. 40	0·750	522
		1. viii. 40	0·647	450
	200	6. i. 40	0·290	202
		12. iii. 40	0·285	198
Zeaxanthin	200†			
	1st batch	3. ii. 40	0·320	200
	2nd batch	27. iii. 40	0·305	191
	3rd batch	7. v. 40	0·301	188
	4th batch	6. vi. 40	0·286	179
	5th batch	1. vii. 40	0·303	189
	6th batch	22. vii. 40	0·285	179

* Fresh unopened bottle.

† Presumptive 'units' based on assumption that 1 mol. zeaxanthin has the activity of 1 mol. of β-carotene.

traces of which were rinsed in with a small quantity of the solvent. The 5 l. flask was finally washed several times with arachis oil and the washings were added to the contents of the 10 l. flask so as to bring the total weight of the solution to 4·38 kg. The last traces of benzene were removed by connecting the flask to a vacuum pump of large capacity while a slow stream of CO_2 from a submerged sintered-glass gas distributor passed through the solution. The temperature of the oil was raised to 40° during the later stages of this operation and care was taken to exclude light. A solution containing 200 I.U./g. for feeding to the rats was prepared from the stronger solution by dilution with arachis oil.

Solution of zeaxanthin. It was intended that some of the pigs should be given quite large doses of zeaxanthin in order that biological activity of a low order could be detected. Attempts were therefore made to prepare rather concentrated solutions in the hope that feeding of excess arachis oil to these pigs could be avoided. Owing, however, to the low solubility of zeaxanthin in oil, we were compelled to use a solution no stronger than 0·0128 %. Such a solution would contain 200 I.U./g. if zeaxanthin had the same activity, molecule for molecule, as β-carotene. We discovered later that zeaxanthin crystallized slowly on storage at room temperature even from solutions as dilute as this. The resulting fall in concentration of the six batches of solution made during the course of the

experiment was estimated colorimetrically by means of a Leitz photometer at intervals during the period in which each batch was fed to the pigs. The loss, which never exceeded 10%, was considered to be negligible. Each batch weighed about 8 kg. and was prepared by a method similar to that used for preparing the carotene solution, except that chloroform was used in place of benzene because the latter is a very poor solvent for zeaxanthin. A portion of the first batch was used for the rat test. The batches were prepared as required in order to avoid long periods of storage.

Storage and stability of the feeding solutions. The solutions were kept under N_2 in a refrigerator. In spite of these precautions some deterioration occurred, as is shown in Table 1.

Levels of feeding

All substances tested were given to pigs and rats at two levels of intake. For pigs the low level was based on the estimates of minimum requirements put forward by Hart [1940], but to find out any differences all substances were fed at the same presumptive level of biological activity.

As the lower dose, 100 I.U. of vitamin A were given daily for 10 lb. live weight. This was equivalent to 7·7 μg. of vitamin A (as alcohol or natural esters) and 13·3 μg. of β-carotene per kg. body weight (*vide supra*). The higher dose for these substances was 300 I.U./10 lb. live weight. Zeaxanthin was presumed to be as active as β-carotene but was fed at levels of 300 and 1500 calculated I.U./10 lb. live weight. Details of rat tests are given separately on p. 703.

Tests on pigs

Methods. A 'normal' pig-meal mixture, similar to that used by us previously [Foot *et al.* 1938; 1939], was prepared for this experiment. It consisted of:

Barley meal	40
Weatings	45
Extracted groundnut cake ...	5
Meat meal	6
Brewer's yeast	2
Ground limestone	$1\frac{1}{2}$
Common salt	$\frac{1}{2}$

Examination showed that the diet contained only traces of yellow pigment and this was mostly xanthophyll. No β-carotene was detected; the carotene content was certainly much less than 0·02 mg. per 100 g.

The general methods of management of the pigs were as already described [Foot *et al.* 1938; 1939].

Three litters of pigs were reared on this ration. The dams of each litter were given the meal shortly after farrowing and received this food alone during suckling. The young pigs were offered 4 parts of the meal mixture together with 1 part of dried skim milk powder from the time they were 3 weeks old; they were weaned when 8 weeks old and the three litters were then pooled, groups from them being selected for five treatments as follows:

Group 1. 2 pigs as controls; no addition to meal mixture.
Group 2. 6 pigs receiving zeaxanthin, 3 the higher and 3 the lower level.
Group 3. 4 pigs receiving β-carotene, 2 the higher and 2 the lower level.
Group 4. 4 pigs receiving vitamin A alcohol, 2 the higher and 2 the lower level.
Group 5. 4 pigs receiving vitamin A ester, 2 the higher and 2 the lower level.

The grouping was carried out in such a way that the litters were as far as possible evenly distributed among treatments. In Fig. 1 and Tables 2 and 3 littermates are indicated by letters.

After grouping, the addition of skim-milk powder was discontinued and the meal alone was fed. Each pig was given in addition once a fortnight a solution of calciferol in oil supplying 500 I.U. of vitamin D daily. The pigs were group-fed twice daily. The amount of food allowed each pen of pigs was based on the live weight of each animal and was adjusted once a week after the animals had been weighed. The standard used was that usually recommended by the Pig Experiments Coordination Committee [Shorrock, 1940]. When appetite was depressed so that the animals in any pen failed to eat the amount demanded by their live weight, they were fed according to appetite.

Once weekly, the pigs were given individually solutions containing their respective addenda. For this purpose, the pigs were placed one at a time in small wooden cages and offered the material mixed with a small quantity of meal. Owing to the large amount of zeaxanthin solution required by the three pigs receiving the higher level they were not separated but were given their zeaxanthin together during one feed each day.

Straw was used as litter in the first part of the experiment and wood shavings during the last few weeks.

Results

Preparation period. Some difficulty was experienced in the rearing and preparation of the experimental pigs. Two litters were reared with no more than normal losses during suckling. In one litter, however, six of the eight pigs alive after the first 27 days died during the suckling period. In most of these post-mortem examination showed inflammation of the intestines and pneumonia. The real cause of the losses was, nevertheless, obscure and there is little reason for attributing them solely to the diet at this early stage.

The vitamin A content of the livers of the animals which died during the preparation period is given in Table 2 (cf. p. 702). The data are few, but they

Table 2. *Vitamin A in livers of pigs lost during the suckling and weaning stage*

	Litter A (Sow 185)	Litter B (Sow 576)				Litter C (Sow 607)			
No. born	12	17				11			
No. alive at beginning of experiment	10	11				8			
				Vitamin A in liver				Vitamin A in liver	
		Age at death days	Wt. liver g.	µg./g.	µg. in whole liver	Age at death days	Wt. liver g.	µg./g.	µg. in whole liver
Losses during preparation period	None	39	154	9·6	1478	18	123	10·8	1328
		74	74	7·8	577	27	223	9·1	2029
		80	151	7·8	1178	32	151	11·3	1706
						49	297	7·0	2079
						54	166	2·8	465
						57	247	4·3	1062
Remaining for grouping	10	8				2			

show that the liver reserves at birth were small and they suggest that the amount was decreasing during suckling. The feeding of carotenoids or vitamin A was started when the pigs, according to litter, were 10 or 12 weeks old, and it is

reasonable to assume that at this point the experimental animals had little or no vitamin A in their livers.

Experimental period

Growth. The growth curves of the twenty experimental pigs for the period of dosing are shown in Fig. 1.

All the pigs receiving vitamin A alcohol and vitamin A ester grew consistently throughout the experimental period. They grew at about the normal rate for pigs receiving good fattening rations and there was no evidence of any advantage of the higher level of vitamin A over the lower.

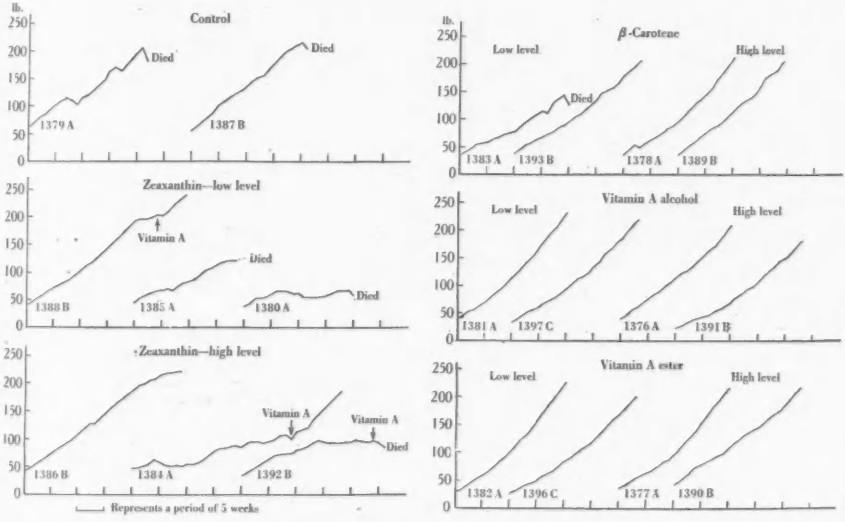

Fig. 1. Growth curves of experimental pigs.

The two animals receiving the higher level of β-carotene also grew consistently and at a good rate. The growth of one on the lower level was, however, definitely subnormal and the animal eventually died 20 weeks after the commencement of the dosing.

The two control pigs were originally the biggest animals from two litters. They continued to grow, although in one case irregularly, for about 20 weeks. Growth then ceased and both pigs died a few days later.

There was much variation in the growth of the pigs receiving zeaxanthin. Two animals, one on the high level and one on the low level, grew normally for about 20 weeks. The first then became steady in weight and did not resume growth until given vitamin A, the second continued to grow at a very reduced rate. The remaining four pigs grew very little throughout the experiment; three of them died, two on the lower and one on the higher level; the fourth recovered after vitamin A treatment.

Health and symptoms. Of the twelve pigs which received vitamin A alcohol, vitamin A ester or β-carotene only one showed any symptoms or evidence of ill-health. This animal (no. 1383) was receiving the low level of β-carotene. Its appetite declined 15 weeks after the start of the experiment. It became thin, failed to thrive, developed laboured breathing and died 5 weeks later without showing any other symptoms.

The two negative control pigs showed no symptoms apart from occasional loss of appetite until the 20th week, when pig no. 1387 experienced a short convulsive fit from which it quickly recovered. The animal, however, became unstable on its legs and died 11 days later following two violent convulsive fits. The appetite of the other control pig, no. 1379, failed completely after the 21st week and the animal was unstable on its feet. Apart from that, however, no symptoms were shown and the pig died suddenly in the 22nd week.

The six zeaxanthin pigs all showed symptoms varying from only slight lack of appetite to typical convulsive fits. One animal, no. 1392, apparently became blind, while another, no. 1388, showed the typical irregular gait which we have often noted in previous experiments [Foot *et al.* 1938; 1939]. Hence the clinical evidence strongly suggested that the zeaxanthin at both the low and the high levels was biologically inactive.

Post-mortem findings. In the six pigs which died during the experiment, pneumonic changes, varying in intensity from slight congestion to grey hepatization, were the most conspicuous findings at necropsy. Patchy inflammation of the intestines, especially of the caecal region, was no less characteristic. This picture agrees with our earlier observations [Foot *et al.* 1938; 1939].

Curative treatment. Three pigs which failed to thrive when their diet was supplemented with zeaxanthin were given curative doses of vitamin A, 1500 I.U./10 lb. body weight daily. A spectacular cure was achieved in two cases (nos. 1384 and 1388); the third pig, which was obviously in a very poor state of health, failed to recover and died a few days after the curative attempt.

Vitamin A content of livers. The livers of all experimental pigs were examined for vitamin A by the method fully described by Foot *et al.* [1938]. The results are given in Table 3 expressed in micrograms of vitamin A. Values in brackets refer to animals which were given vitamin A as a curative measure. As a result of several estimations which agreed well together we have found that 1 Moore blue unit [Moore, 1930], as measured by us on vitamin A concentrates or on the dilutions used for pig feeding, is equivalent to 0·137 μg. vitamin A. The values are quoted on the assumption that the same factor would apply to the vitamin A present in pig livers. Dr A. E. Gillam kindly undertook to assay the livers by the spectrographic method. Unfortunately the results were with two exceptions rendered inconclusive because the vitamin peak was obscured by irrelevant absorption. Good vitamin A curves, however, were obtained for the livers of pigs nos. 1377 and 1388, especially for the former. From the extinction coefficients at 328 mμ it was calculated that these livers contained 22·3 and 139 mg. of vitamin A respectively. The corresponding figures calculated from the blue value measurements were 25·6 and 72 mg.

The complete absence of vitamin A from the livers of the three pigs which received only zeaxanthin is of particular interest and confirms the clinical evidence that this carotenoid is biologically inactive. The livers of the two pigs receiving the lower dose of β-carotene contained little or no vitamin A while the reserves of those given 300 I.U. of β-carotene per 10 lb. live weight were rather less than those of pigs given only 100 I.U. as vitamin A alcohol or ester.

Table 3. *Vitamin A in livers of experimental pigs*

Pig no. and litter	Age at death days	Wt. of liver g.	Vitamin A in liver	
			μg./g.	μg. in whole liver
Control 1379 A	249	1889	0	0
1387 B	229	2604	0	0
Zeaxanthin (low level)				
1380 A	233	923	0	0
1385 A	248	1505	0	0
1388 B	290	2009	(36·0)*	(72,324)*
Zeaxanthin (high level)				
1384 A	359	1501	(4·5)*	(6,754)*
1386 B	290	1500	0	0
1392 B	269	1057	(2·3)*	(2,431)*
Vitamin A ester (low level)				
1382 A	238	2094	1·3	2,722
1396 C	247	1698	1·3	2,207
Vitamin A ester (high level)				
1377 A	238	1684	15·2	25,597
1390 B	248	1698	8·8	14,942
β-Carotene (low level)				
1383 A	233	1796	0	0
1393 B	248	1698	Faint trace	Trace
β-Carotene (high level)				
1378 A	238	1953	0·5	976
1389 B	227	1698	0·3	509
Vitamin A alcohol (low level)				
1381 A	238	2066	1·4	2,892
1397 C	247	1868	0·9	1,681
Vitamin A alcohol (high level)				
1376 A	238	1670	9·1	15,197
1391 B	248	1613	7·8	12,581

* After receiving vitamin A.

Tests on rats

The substances given to pigs were also assayed on rats in curative tests. The technique was as described by Booth *et al.* [1934], with the minor modifications that dextrinized potato starch was used instead of dextrinized rice starch, that de Loureiro's [1931] salt mixture was used and that a small fraction of the yeast present in the diet was irradiated to supply vitamin D. The tests were of 4 weeks' duration. All test substances were given twice weekly, β-carotene, vitamin A alcohol and vitamin A ester at two levels of 2 and 4 I.U. daily and zeaxanthin at the presumptive levels of 4 and 12 I.U. (cf. p. 699). Groups of four male and five female rats were used for each substance. The results are given in Table 4.

Only three out of nine animals receiving the lower level of zeaxanthin survived the experimental period. There were two deaths at the higher level and the remaining seven rats lost weight heavily. The results show that zeaxanthin is of no value for the rat as provitamin A.

All rats receiving vitamin A alcohol, vitamin A ester and β-carotene survived, and gains in weight at corresponding levels of intake were very similar, indicating that all three substances had the same biological activity.

Table 4. *Weight gains of vitamin A-deficient rats receiving various supplements*

Zeaxanthin							
4 I.U.				12 I.U.			
		4 weeks				4 weeks	
Rat no.	Initial wt. (g.)	Wt. (g.)	Gain (g.)	Rat no.	Initial wt. (g.)	Wt. (g.)	Gain (g.)
9877	110	Dead		9872	87	64	−23
9894	66	Dead		9901	85	65	−20
9902	78	Dead		9909	86	70	−16
9932	94	110	16	9933	90	97	7
9869	112	Dead		9876	94	Dead	
9881	63	Dead		9882	58	54	− 4
9905	76	Dead		9915	64	60	− 4
9922	136	108	−28	9926	116	90	−26
9929	98	85	−13	9935	71	Dead	
Vitamin A alcohol							
2 I.U.				4 I.U.			
9885	51	66	15	9886	65	89	24
9910	94	106	12	9892	86	115	29
9919	81	93	12	9924	66	97	31
9939	66	82	16	9941	66	90	24
Mean ♂♂	73·0	86·8	13·8		70·8	97·8	27·0
9873	100	116	16	9874	73	84	11
9890	82	91	9	9898	64	76	12
9896	77	89	12	9906	74	87	13
9914	73	81	8	9928	96	105	9
9938	72	79	7	9937	65	80	15
Mean ♀♀	80·8	91·2	10·4		74·4	86·4	12·0
Mean ♂♂ and ♀♀	77·3	89·2	11·9		72·8	91·4	18·6
β-Carotene							
2 I.U.				4 I.U.			
9879	101	112	11	9880	98	124	26
9903	101	114	13	9884	67	85	18
9908	82	99	17	9895	100	139	39
9918	73	90	17	9917	82	106	24
Mean ♂♂	89·3	103·8	14·5		86·7	113·5	26·8
9868	110	110	0	9870	92	110	18
9883	63	70	7	9904	81	100	19
9889	82	103	21	9913	66	88	22
9897	96	109	13	9921	116	125	9
9920	127	139	12	9936	78	78	0
Mean ♀♀	95·6	106·2	10·6		86·6	100·2	13·6
Mean ♂♂ and ♀♀	92·8	105·1	12·3		86·7	106·1	19·4
Vitamin A ester							
2 I.U.				4 I.U.			
9887	66	74	8	9878	67	86	19
9893	74	86	12	9888	54	64	10
9900	70	88	18	9911	95	113	18
9934	107	131	24	9931	92	118	26
Mean ♂♂	79·3	94·8	15·5		77·0	95·3	18·3
9871	99	104	5	9867	111	121	10
9875	84	96	12	9899	80	105	25
9891	96	111	15	9912	70	96	26
9907	78	84	6	9923	85	106	21
9916	72	87	15	9930	108	113	5
Mean ♀♀	85·8	96·4	10·6		90·8	108·2	17·4
Mean ♂♂ and ♀♀	82·9	95·7	12·8		84·7	102·4	17·7

Discussion

The results show without doubt that the pig, like the rat, is unable to make use of a hydroxy-β-ionone ring and cannot utilize zeaxanthin. Lund's [1938] results to the contrary were most probably due to active impurities present in his

preparation of zeaxanthin. It is possible that the slight difference between the behaviours of the rats receiving in our experiments the two levels of this carotenoid may have been due to traces of carotene or cryptoxanthin retained in our preparation in spite of the precautions taken to secure a pure specimen.

A striking result of the present work is the confirmation of the findings of Guilbert *et al.* [1937] that the pig utilizes carotene less efficiently than vitamin A even under conditions probably not far removed from optimal. The two pigs receiving 100 I.U. of β-carotene per 10 lb. live weight were unable to store any vitamin A in their livers. One of them died and the post-mortem findings were typical of vitamin A deficiency. The two pigs receiving 300 I.U. of β-carotene grew satisfactorily, but their liver reserves were slightly lower than those of pigs given only one-third of this quantity as preformed vitamin A. Making due allowance for the small number of pigs used in our experiments we feel it safe to suggest that for the pig the efficiency of conversion of β-carotene into vitamin A is at best no greater than 30–40 %. On the other hand previously depleted rats utilized in our experiment β-carotene as efficiently as preformed vitamin A. The dosages were comparable with those received by the pigs, as 2 and 4 I.U. daily corresponded in this case to about 100 and 200 I.U./10 lb. live weight (Table 4). Though the treatment was preventive for pigs and curative for rats, both species were receiving similar levels of the provitamin dissolved in the same oil and the diets were also similar in being low in fat. It would seem therefore that the difference is a true one in metabolic behaviour.

The findings of Guilbert and his colleagues [Hart, 1940] point in the same direction and there are also indications that carnivora utilize carotene inefficiently as a source of vitamin A [Ahmad, 1931; Rea & Drummond, 1932; Frohring, 1935; cf., however, Bradfield & Smith, 1938]. Whatever the species, the ratio of the respective efficacies of vitamin A and carotene widens as the dose is increased [Guilbert *et al.* 1937; 1940]. Davies & Moore [1934] have reported that even the rat assimilates vitamin A at high levels much better than carotene. The careful tests of Wagner [1940] show the difference in the utilization of the provitamin and of vitamin A itself by man even after vitamin A deprivation. His findings are supported by numerous other reports, of which those of Booher *et al.* [1939], Booher & Calliston [1939] and Kreula & Virtanen [1939] may be quoted as examples. All these observations certainly bring out the complications and difficulties inherent in the use of carotene as a standard for vitamin A and make the suggestion of Guilbert *et al.* [1940] that separate standards for vitamin A and for carotene should be adopted worthy of serious consideration.

If the definition put forward by Guilbert *et al.* [1937] that the minimum level of vitamin A is one producing good growth but little storage in the liver is accepted, then our results are in good agreement with their findings. In our case the conditions were satisfied by 7·7 μg. of vitamin A as the alcohol or its natural esters and 40 μg. of β-carotene daily per kg. body weight. Figures in Table 1 show that the solutions of vitamin A alcohol and vitamin A ester deteriorated slightly during the tests. The loss of potency was of the order of 5 % and if taken into consideration would reduce the estimate of the minimum requirements to 7·3 μg. The figures given by the American workers for vitamin A and carotene were respectively 5·1–6·7 μg. and 25–39 μg.

The results of the rat assay of the vitamin A esters and vitamin A alcohol reported by us confirm the conclusions reached independently by Underhill [unpublished work] that there is no difference in the effect on the growth of vitamin A-deficient rats of equal quantities of vitamin A alcohol or natural vitamin A esters, when these quantities are estimated by measurement of the

spectrographic absorption at 328 mμ. The significance of this result will be fully discussed by Underhill, so that it is only necessary to point out here that its attainment was probably due to the freedom of the preparations tested from oxidation products and other impurities which interfere with the spectrographic estimation.

The evidence as to the relative efficacies of free and esterified vitamin A for pigs fully supports the results obtained on rats, but owing to the use of fewer animals it carries less weight. There seems nevertheless to be some justification for the hypothesis that the two forms of the vitamin are equally, or nearly equally, effective for pigs.

Summary

1. The vitamin A activities of carefully purified preparations of zeaxanthin, vitamin A alcohol, vitamin A ester and β-carotene were assayed on pigs and rats.

2. Zeaxanthin was biologically inactive for both species.

3. Pigs made less efficient use of β-carotene than rats and required, to cover their minimal requirements, about 300 I.U. of β-carotene or 100 I.U. of preformed vitamin A daily per 10 lb. live weight.

4. No difference was found in the biological activities of vitamin A alcohol and vitamin A natural ester for either pigs or rats.

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74. METABOLISM OF STEROIDS

2. THE ISOLATION OF CHOLESTANE-3:5:6-TRIOL AND OTHER SUBSTANCES FROM OX LIVER EXTRACTS

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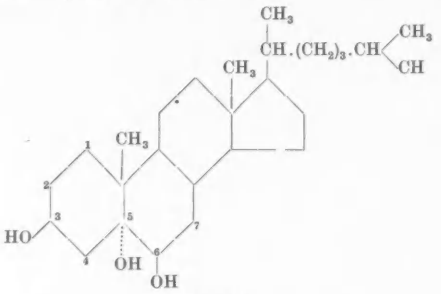
In Part I [Haslewood, 1939], the isolation from ox liver of α-7-hydroxycholesterol was reported. Since that time, a 'β-7-hydroxycholesterol' has been mentioned as isolated from hog liver [MacPhillamy, 1940], and β-7-hydroxycholesterol has been obtained from pregnant mares' serum [Wintersteiner & Ritzmann, 1940]. Wintersteiner & Bergström [1941] report further that cholesterol can be converted into a mixture containing 7-hydroxy- and 7-keto-cholesterol by treatment at an alkaline reaction with oxygen at 85°. As Wintersteiner and his colleagues ably and correctly point out, all this work does not answer the question of the authenticity of the oxygenated cholesterols as metabolites, since it would seem possible that such oxidation products are formed from cholesterol during the processes leading to their isolation. Oxidation of cholesterol appears to take place readily at the double bond, as well as at position C_7, as would be expected on chemical grounds, but whether the products are actually those taking part in metabolism can only be decided by many types of experiment, including, perhaps, such work as the treatment of cholesterol with enzymic preparations.

The present work was carried out on the same fraction of ox liver as was previously used, and from this source there has been obtained an incompletely purified *alcohol*, probably $C_{24}H_{46}O_3$, characterized as the *monoacetyl derivative*, $C_{24}H_{45}O_2(O.CO.CH_3)$. This substance is not of the steroid type.

α-7-Hydroxycholesterol (identified as the dibenzoate) has been obtained from the extract directly as crystals melting at 174–176°: the compound is difficult to crystallize [cf. Wintersteiner & Ritzmann, 1940].

There has also been isolated from the liver extract cholestane-3:5:6-triol, identified as the diacetate. This triol is obtained by oxidation of cholesterol or its acetate with H_2O_2 [Pickard & Yates, 1908] and has been called 'triol I' by Ellis & Petrow [1939], who assign to it the structure:



Many authors have stressed the analogy between biological oxidations and those brought about by H_2O_2; nevertheless the foregoing remarks about the origin of substances isolated apply to cholestane triol, especially since ether was used in its preparation from liver.

As already reported [MacPhillamy, 1940; Haslewood, 1941], the 'hepatols' have been derived from digitonin used in their preparation. One of them, 'hepatol A', is apparently digitogenin, $C_{27}H_{44}O_5$, as stated by MacPhillamy [1940]. Its confusion with a C_{21} compound arose because of the difficulty of obtaining an accurate measurement of its molecular weight. The problem was solved when the diacetate was oxidized with cold chromium trioxide to a monocarboxylic acid, probably $C_{30}H_{45}O_7.COOH$, which was titrated. This acid was obtained in good yield; its formation is explicable in terms of the formula assigned to digitogenin by Marker & Rohrmann [1939], although it is difficult to understand why digitogenin (with hydroxyl groups at C_2, C_3 and C_6) should not easily be completely acetylated.

The cholestane triol was first obtained as the digitonide. This was decomposed with pyridine and acetic anhydride and from the acetylated mixture the diacetate of the triol was readily crystallized.

Experimental

Analyses were microanalyses by Dr A. Schoeller (S) and Dr G. Weiler (W). All melting points are uncorrected.

Starting material was the evaporated methyl-alcoholic mother liquors (fraction A) from the crystallization of the 'sterols' from the unsaponifiable ether-soluble fraction of ox-liver 'marc' [Haslewood, 1941].

The alcohol (?) $C_{24}H_{46}O_3$. Fraction A (20 g.), on standing in solution in about 20 ml. of ether, or ether in light petroleum, slowly deposited a semi-crystalline solid (0·1 g.) which was collected and crystallized from acetone. It formed colourless globules of small crystals, M.P. 93–95°, not soluble in alkali, readily forming a 'gel' from dilute alcohol and giving negative tests for N and P. The substance gave no precipitate with digitonin or with 2:4-dinitrophenylhydrazine in dilute alcoholic solution. Found (W): C, 74·85, H, 12·4%, mol. wt.=353. $C_{24}H_{46}O_3$ requires C, 75·4, H, 12·0%, mol. wt.=382.

This material (50 mg.) in pyridine (0·2 ml.) with acetic anhydride (0·2 ml.) was heated at 100° for 15 min. The precipitate collected after dilution with water was recrystallized from acetone and separated as clumps of white needles, M.P. 103–105°. Found (W): C, 73·8, H, 11·3%, mol. wt.=428. $C_{24}H_{45}O_2(O.CO.CH_3)$ requires C, 73·6, H, 11·3%, mol. wt.=424. A sample from the mother liquors had M.P. 107–108°, while another obtained after six crystallizations from acetone of the 'sterols', acetylated with acetic anhydride, melted at 100–111°, both samples being saponified with dilute alcoholic NaOH to the above alcohol. These facts suggest that the material has not been completely purified, though all samples of the acetate had sharp melting points.

Separation of hydroxylated cholesterols. Fraction A (16 g.) was dissolved by shaking with 100 ml. of 90% (by vol.) aqueous methyl alcohol and 100 ml. of light petroleum (B.P. 40–60°). The alcoholic layer was separated and washed in a funnel with two portions each of 50 ml. of fresh petroleum. The combined petroleum on evaporation gave 14·3 g. of *fraction B*, while evaporation of the alcohol yielded *fraction C* (1·7 g. of an orange gum). Fraction C was dissolved in 25% (by vol.) benzene in light petroleum and allowed to run through a cylindrical column (length 25 cm., diam. 0·8 cm.) of Al_2O_3 (Hopkin & Williams).

The column was then washed with solvents as follows, the eluates being collected separately and evaporated, and the weights of the residues being obtained. In this way, fractions were collected as below:

Fraction	Solvent	Weight g.	Description of residue
I	Up to 100 ml. of 25% benzene in light petroleum	0·2	Mobile oil
II	100 ml. of benzene	0·15	,,
III	100 ml. of ether	0·8	Stiff gum
IV	100 ml. of warm alcohol	0·2	,,
V	100 ml. of alcohol acidified with HCl and warmed with the disintegrated column; product isolated with ether, after dilution	0·17	,,

Fraction III (0·8 g.) in benzene was adsorbed on a fresh column (20 × 0·8 cm.) and the column eluted with 100 ml. of 50% (by vol.) benzene in ether (residue (0·55 g.): fraction III*a*) and then with 100 ml. of alcohol (residue (0·25 g.): fraction III*b*).

Fraction III a (0·55 g.) in benzene was adsorbed on a fresh column (10 × 0·8 cm.) and was eluted with benzene up to 500 ml. Evaporation of the solvent gave solid α-7-hydroxycholesterol, which, from benzene in light petroleum, formed long white needles, M.P. 174–176° decomp. Yield, ca. 10 mg. 5 mg. of this product on benzoylation with PhCOCl (1 drop) and pyridine (2 drops) for 2 hr. at room temperature gave on dilution a gum which was crystallized from methyl alcohol to give white needles of α-7-hydroxycholesterol dibenzoate, M.P. 170–171°, not depressed by authentic material, M.P. 170–172°. The hydroxycholesterol and its dibenzoate gave the characteristic colour and precipitation reactions.

Fraction III b (0·25 g.) was warmed with a solution of digitonin (0·2 g.) in 20 ml. of 80% alcohol. A crystalline digitonide separated rather slowly and after 24 hr. was collected, washed with alcohol and ether and dried. The product (50 mg.) in pyridine (0·5 ml.) with acetic anhydride (0·5 ml.) was heated for 20 min. at 100°. The precipitate obtained after dilution with water was collected and dissolved in warm alcohol. When the warm filtered solution was diluted to faint cloudiness and allowed to stand, needles separated and were collected and recrystallized from dilute alcohol, from which separated long glistening needles (8 mg.), M.P. 163–165°, not depressed by cholestane triol I diacetate, M.P. 167–168°, prepared by acetylation by the above method of the purified triol. Found (W): C, 73·4, H, 10·6%, M = 453, 480. $C_{31}H_{52}O_5$ requires C, 73·8, H, 10·3%, mol. wt. = 504. Saponification gave cholestane triol (M.P. 235–237°) identical with authentic material. 40 mg. of authentic triol (M.P. 233–235°), obtained from cholesterol with H_2O_2, together with 0·1 g. of digitonin, were dissolved by warming with 15 ml. of 80% alcohol. After standing for 24 hr., the crystalline digitonide (80 mg.) was collected, washed, dried and acetylated as above. The acetate obtained had M.P. 163–165°, not depressed by the specimen from liver.

'*Hepatol A*' *from digitonin.* Digitonin (0·2 g.), with a few drops of a dilute solution of Br_2 in alcohol, was refluxed for 2 hr. with 2 ml. of xylene. The xylene was evaporated and the residue purified by washing with light petroleum, sublimation and crystallization as previously described. White needles, M.P. 275–278°, not depressed by 'hepatol A', were obtained.

The acid $C_{30}H_{45}O_7.COOH$. The diacetate (10 mg. of M.P. 235–238°, and prepared as previously described) of the above compound was dissolved in glacial acetic acid (0·2 ml.) and the mixture allowed to stand at room temperature for 16 hr. with 0·2 ml. of a solution made by dissolving 10 g. of CrO_3 in the minimal amount of water and making up to 100 ml. with acetic acid. After dilution with water, the precipitate was collected and recrystallized from dilute alcohol, from which it formed white needles, M.P. 263–264°, decomp. Yield, 7 mg. Found (S): C, 65·7, H, 8·2%, mol. wt. (titration) = 540, 519. $C_{30}H_{45}O_7.COOH$ (one carboxyl group) requires C, 66·2, H, 8·2%, mol. wt. = 562. The above compound was easily soluble in warm dilute $NaHCO_3$ solution, from which it was precipitated on acidification. It showed no alteration after treatment with acetic anhydride and pyridine and gave no obvious reactions with ketone-detecting substances. With diazomethane, a partially purified methyl ester, M.P. 184–186°, was formed. Found (S): C, 66·3, H, 8·5%. $C_{30}H_{45}O_7.COOCH_3$ requires C, 66·7, H, 8·3%.

Summary

In addition to crystalline α-7-hydroxycholesterol, M.P. 174–176°, there has been obtained from the unsaponifiable fraction of an ether extract of ox liver cholestane-3:5:6-triol I, and an alcohol, M.P. ca. 95°, characterized as a monoacetyl derivative. 'Hepatol A' is digitogenin: its diacetate has been oxidized to a monocarboxylic acid.

Experiments of the type now reported do not finally establish the authenticity of isolated substances as intermediates in metabolism.

The author's sincere thanks are due to Messrs Boots Pure Drug Co. for extracting and saponifying a large quantity of ox liver marc, and also to Prof. R. A. Peters for generous hospitality accorded at the Biochemical Laboratories, Oxford.

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75. THE STEROL REQUIREMENTS OF THE LARVA OF THE BEETLE, *DERMESTES VULPINUS* FABR.

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Hobson [1935] showed that blowfly larvae required a fat-soluble growth factor which was contained in the unsaponifiable ether-soluble fraction of their food and proved to be identical with or replaceable by cholesterol. Subsequently the indispensability of cholesterol for the growth of many insects was demonstrated (Van t'Hoog [1936] for *Drosophila*; Chiu & McCay [1939] for the bean weevil *Acanthoscelides obtectus*; Fröbrich [1939] and Offhaus [1939] for the flour beetle *Tribolium confusum*; and for the edible snail, *Helix pomatia*, Howes [1937]). In a study of the nutritional requirements of the beetle *Dermestes vulpinus*, carried out in this department, Gay [1938] showed that growth of the larva was satisfactory on a diet consisting of casein, cystine, yeast, salt mixture and cholesterol, or of yeast, salts and cholesterol, while omission of cholesterol from either diet prevented growth. It has been the purpose of the present investigation to obtain more detailed information about the sterol requirements of this beetle.

Experimental

The technique of breeding was very similar to that employed by Gay [1938]. The method of obtaining eggs and newly hatched larvae has been described by Dick [1937]. The insects were bred at 27° and 70% relative humidity except in the last series of experiments (Tables 5–7), when the temperature was 25°. It was at first thought that the larvae would attack each other and to prevent such loss of animals by cannibalism they were bred singly in glass tubes, the history of growth of each larva being followed, as a rule by daily inspection, until pupation or death. This precaution, however, later proved unnecessary and in the last series five larvae were reared together in a tube.

The following diets were used:

Diet I (called subsequently 'casein diet'): 3·0 g. ashless vitamin-free casein (casein E, Glaxo Laboratories), 0·3 or 0·15 g. dried debittered brewer's yeast (Glaxo Laboratories), 0·1 g. sterol, 0·02 g. cystine (B.D.H.), 0·03 g. MacCollum's salt mixture, distilled water to 50% by weight.

Diet II (called subsequently 'yeast diet'): 3·0 g. dried debittered brewer's yeast (Glaxo Laboratories), 0·1 g. sterol, 0·03 g. MacCollum's salt mixture, distilled water to 50% by weight.

Diet III (described on p. 713).

The following sterols were tested, their source being indicated in the list:

From Glaxo Laboratories: cholesterol, sitosterol, ergosterol, calciferol, 7-keto-cholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate, 7-dehydrocholesteryl monobenzoate, 7-dehydrocholesterol.

From Prof. I. M. Heilbron and Dr E. R. H. Jones: cholesterol purified via the dibromide, cholestanol (dihydrocholesterol), zymosterol, Δ^5-cholestene, cholesteryl acetate.

At a temperature of 27 or 25°, a humidity of 70% and a water content of 50% the diet would be expected to become infested with moulds and other micro-organisms within a few days. This might seriously alter the composition of the food, apart from introducing new food factors supplied by the micro-organism. Serious efforts were made to limit the degree of infestation and four different methods were tried for this purpose.

1. An attempt was made to grow the larvae under sterile conditions. The eggs were sterilized by immersion in a 3% solution of $HgCl_2$ for 3 min. The food was sterilized by heating the dry constituents, minus the yeast, at 145° for 1½ hr., and to them was added a suspension of yeast in water which had been brought to the boil. Two days later this mixture was again heated at 100° for 10 min. While the cultures seemed to remain sterile for about 12–16 days, almost invariably moulds then began to grow, most probably due to infestation from outside. Although it seemed likely that lasting sterility could be achieved by further improvement of the technique the drawbacks of the method were so manifold that it was finally abandoned.

2. During the past few years increasing use has been made of the antiseptic properties of the esters of *p*-hydroxybenzoic acid for keeping organic media free from bacteria and moulds [e.g. Cremer, 1935]. The methyl ester, known under the name 'Nipagin M', is used in this connexion in the breeding of *Drosophila*. We found in a series of experiments that admixture of 0·5% of Nipagin in the wet casein diet prevented growth of moulds in a varying proportion of the cultures while having no influence on the growing larvae. On the other hand, moulds never appeared in a yeast diet to which 0·5% Nipagin had been added. In spite of the uncertain effect of Nipagin as a fungicide, useful results could be obtained with this method.

3. The food was changed daily to prevent the development of moulds. This method was previously used by Gay [1938] and was adopted for the first of the present series of experiments. Although it yields very reliable results it has the disadvantage of being very laborious and requiring large amounts of the diets.

4. After many of the experiments had been completed the following diet was devised, based on the strong hygroscopic properties of fructose:

Diet III (called subsequently 'yeast fructose diet'): 1·5 g. dried debittered brewer's yeast (Glaxo Laboratories), 1·5 g. fructose, 0·05 g. sterol (or less), 0·03 g. MacCollum's salt mixture (not essential and usually omitted), 0·4 ml. water. This quantity was enough for five larvae, which were reared together in one tube.

Owing to the hygroscopic properties of fructose the diet, under the conditions at which it was used (25° and 70% relative humidity), acquired a water content of 20 to 25%, and the high sugar content prevented growth of moulds very effectively. One of the minor disadvantages of the diet was that the water content was not exactly the same in different samples, and since in *Dermestes* the rate of growth is very much dependent on the water content of the diet [Gay, 1938] any minor differences which were recorded in the duration of larval life could not be regarded as significant. An attempt to breed *Dermestes* on a casein diet to which 50% fructose was added proved less successful because the diet got so wet that many young larvae were drowned in it.

In all experiments except the later ones on the fructose diet, the development of each individual larva was observed up to the time of pupation, and the subsequent emerging of the adult and the number and time of each moult were recorded. The data presented as a measure of the efficiency of a diet are limited in this paper for the most part to the times until the larvae pupated or, in case of an unsuccessful diet, died. The duration of larval life is the only figure of

significance, since growth takes place only during that period. Once a larva has pupated, the duration of the pupal stage is constant (about 7 days at 27°). The adults were always fertile. The controls were reared on fishmeal which was moistened to contain 50% water.

The first question to be decided was whether the positive effect of cholesterol on growth was not due to the presence of impurities such as 7-dehydrocholesterol, the natural provitamin D_3 which is considered to be present in commercial cholesterol. Series I compares the effect of commercial cholesterol (B.D.H.) with a sample of cholesterol specially purified (via the dibromide), which was free of 7-dehydrocholesterol. Although for reasons not connected with the course of the experiment this series had to be abandoned after 11 days, it was by then clear that the larvae grown on the two samples of cholesterol hardly differed with regard to mortality and speed of development and were slightly more advanced in development than the controls (Table 1). In the absence of cholesterol, development was slow, the mortality was high and the few surviving larvae were in an obviously dying condition.

Table 1

No.	Diet	Condition after 11 days
1	Fishmeal (control)	9 in 4th instar 1 dead
2	Yeast diet, commercial cholesterol	9 in 5th instar 1 dead
3	Yeast diet, purified cholesterol	6 in 5th instar 0 dead 4 in 4th instar
4	Yeast diet, no sterol	2 in 3rd instar 7 dead 1 in 4th instar

Temp. 27°. Food changed daily.

Series II (Table 2) was devised to compare the effect of purified cholesterol with those of sitosterol, ergosterol and calciferol. The first three sterols were incorporated in a casein diet containing 5% yeast and the food was changed daily. This method was not used with calciferol, owing to the expense involved; the Nipagin method was used instead.

It can be seen in Table 2, nos. 7–9, that the diets containing ergosterol or calciferol were entirely inadequate. In fact the larvae died as rapidly as if no sterol had been present [cf. Gay, 1938]. Sitosterol proved of slight nutritional

Table 2

No.	Diet	Method	No. of larvae	No. pupating	Mean time to pupation days
5	Pure, cholesterol, casein diet, 5% yeast	Food changed daily	10	5	39·8
					Mean time to death days
6	Sitosterol, casein, diet, 5% yeast	Food changed daily	10	Last larva died after 30 days (one pupated 47th day)	18·2
7	Ergosterol, casein diet, 5% yeast	Food changed daily	10	Nil. Last died after 13 days	10·4
8	Calciferol, casein diet, 5% yeast	0·5% Nipagin; 3 tubes grew mould 11th day	15	Nil. Last died after 15 days	12·3
9	Calciferol, yeast diet	0·5% Nipagin; no mould	15	Nil. Last died after 14 days	10·1

Temperature 27°.

value. Only one larva reached the pupal stage after long delay and the remaining larvae lived considerably longer than on ergosterol and calciferol. The course of the experiment on pure cholesterol was not entirely satisfactory. The mortality was somewhat higher than it should have been and the time to pupation of 39·8 days compares unfavourably with Gay's figure of 29·2 days, which was obtained, however, in experiments with commercial cholesterol.

Series III (Table 3) was devised to compare the efficiencies of commercial and pure cholesterol. To make the general conditions more favourable a casein diet with 10 % (instead of 5 %) of yeast was used. It was desirable to know whether any fat-soluble factors in yeast other than sterols would play a role in the

Table 3. *Series III. Casein diet with 10 % yeast. 0·6 % Nipagin added; food changed once on the 15th day*

No.	Diet	No. of larvae	No. pupating	Mean time to pupation, days
10	Commercial cholesterol	15	11	26·0
11	Pure cholesterol	15	11	26·0
12	Commercial cholesterol ether-extracted yeast	15	11	29·2
13	Pure cholesterol ether-extracted yeast	15	14	28·6
			Last died after, days	Mean time to death, days
14	Ergosterol	15	31	15·8
15	Sitosterol	15	34	20·0

Temperature 27°.

nutrition of *Dermestes*. The effect of ether-extracted yeast (extracted for 7 days at room temperature, ether changed once or twice daily) was therefore compared with that of ordinary yeast. The series was set up with the Nipagin method, and in order to obviate possible water loss from the diet it was changed once on the 15th day. In order to make the conditions strictly comparable for all the sterols used further tests with ergosterol and sitosterol were included.

Comparing nos. 10 and 11 and nos. 12 and 13 it is obvious that there is no difference in the effects of commercial and of pure cholesterol. It is therefore clear that the effective substance for the growth of *Dermestes* is cholesterol and not 7-dehydrocholesterol. It is not likely that any ether-soluble constituents of yeast are required. The slight prolongation of larval life on a diet containing ether-extracted yeast is not considered to be significant and may well be due to traces of the ether remaining in the yeast. Ergosterol and sitosterol again had no effect. Growth was very slow and all larvae died long before reaching the state of pupation, although single larvae remained alive for a considerable period. The longer survival on sitosterol and ergosterol and the quicker rate of development on cholesterol seen in Table 3, compared with those seen in Table 2, are almost certainly due to the larger amount of yeast in the diet.

In the experiments in which the food was mixed with Nipagin and was not changed daily some growth of mould usually occurred. In some tests only a small proportion of the tubes developed mould, in others a large proportion, though in none was the growth strong and it was always delayed in appearance. The data given in Table 4 show that the slight growth of mould that occurred had no significant effect upon the results of the experiments. In most instances the mean time until death or pupation of all larvae was not significantly different

Table 4. *Casein diets (except no. 9)*

No.	Medium	No. of tubes	No. with mould	Mean time to appearance of mould days	Mean time to death or pupation of all larvae days	Mean time to death or pupation of those exposed to mould days	
9	Calciferol, yeast diet, 0·5% Nipagin	15	0	—	10·1	—	Time to death
8	Calciferol, 5% yeast, 0·5% Nipagin	15	3	10·0	12·3	12·0	
15	Sitosterol, 10% yeast, 0·6% Nipagin	15	5	12·0	20·0	17·4	
14	Ergosterol, 10% yeast, 0·6% Nipagin	15	2	19·0	15·8	20·0	
11	Specially purified cholesterol, 10% yeast, 0·6% Nipagin	15	13	11·0	26·0	25·8	Time to pupation
10	Commercial cholesterol, 10% yeast, 0·6% Nipagin	15	10	9·0	26·0	25·8	
13	Specially purified cholesterol, 10% ether-extracted yeast, 0·6% Nipagin	15	5	16·6	28·6	29·2	
12	Commercial cholesterol, 10% ether-extracted yeast, 0·6% Nipagin	15	9	10·8	29·2	31·8	

from the mean times of those alone in which mould occurred. In only one case (no. 15) did the mould seem to have an adverse effect and in only one case (no. 14) a beneficial effect.

Long after the experiments so far described had been concluded the new yeast fructose technique, described on p. 713, was devised and new series of experiments were set up (Tables 5 and 6). In view of the large amounts of yeast present (50% of dry diet) it was not considered essential to add salts. In fact, addition of salt mixture did not alter the result if cholesterol or any other growth-promoting sterol was present. When, however, no sterols were supplied, the addition of salts usually had the effect of delaying death in a few individuals. As a consequence no salts were added in the experiments of Table 6 except where expressly stated (no. 21).

It is obvious from Table 5, nos. 16 and 17, that the activities of pure cholesterol and 7-dehydrocholesterol are about equal, although smaller quantities of the latter were used. The periods required for pupation in this series are considerably longer than in those of former experiments (Table 3). This is due mainly to the lower temperature of breeding (25 against 27°) and the lower water content (20–25 against 50%); it may also have been due in part to the lower protein content of the fructose diet and the breeding of five larvae together in one tube, instead of single cultures. On the other hand, the lower death rate in the fructose diet experiments suggests that the conditions must have been more favourable in other respects.

Of thirteen sterol preparations tested (Tables 5 and 6) only four proved to be of any nutritional value: cholesterol, dehydrocholesterol and two esters of these substances, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate.

Table 5. *Yeast fructose diet; 50 mg. of sterol (10 mg. in case of dehydrocholesterol) in 3 g. of food; 25°*

No.	Sterol	No. of larvae	No. of pupae	First pupa at day	Last pupa at day	Average time of pupation days
16	Cholesterol	10	10	33	39	36
17	7-Dehydrocholesterol, 10 mg.	10	10	33	38	35·5
18	Cholesteryl acetate	10	10	32	41	36·5
19	7-Dehydrocholesteryl monobenzoate	10	8	33	39	36

Table 6. *Yeast fructose diet; 50 mg. of sterol in 3 g. of food; 25°*

No.	Additions to diet	No. of larvae	First dead at day	Last dead at day	Average time of death days
20	No sterol, no salts added	30	16	22	19
21	No sterols, salts added	10	16	53*	24
22	Sitosterol	20	18	57*	23·4
23	Ergosterol	20	16	25	20·5
24	Zymosterol	20	16	18	16·5
25	Chloroform extract of wholemeal flour	20	16	21	18·5
26	Cholestanol	20	16	21	18·5
27	Cholestene	10	17	37* (1 pupa 53 days)	21
28	7-Ketocholesteryl acetate	20	16	48*	19·8
29	7-Hydroxycholesterol	20	16	40*	18·8
30	7-Hydroxycholesteryl dibenzoate	20	16	40* (1 pupa 42 days)	19

* One larva only survived for any considerable time.

The differences in the average survival times are not considered to indicate significant differences in the potencies of the inadequate diets. It was not considered advisable to disturb cultures too often, and at the first inspection usually several larvae were dead, so that the figures of the last column cannot be regarded as accurate mean values. The fact that on several diets one larva survived the others for a considerable time, and on cholestene and 7-hydroxycholesteryl dibenzoate even reached pupation, may be of some significance, but is more likely due to the presence of impurities in the sterols. This is the more probable since the chloroform-soluble fraction of whole wheat, which besides sitosterol certainly contained mixtures of other sterols and which proved to be an adequate source of sterols for several flour insects (unpublished), was entirely negative.

This also raised the question of whether some of the positive effects of sterols (Table 5) may not equally be due to impurities. The preparations which were available, with the exception of cholesterol, were admittedly not chemically pure, and it was possible that the presence of cholesterol in 7-dehydrocholesterol and in 7-dehydrocholesteryl monobenzoate might be the cause of their being of positive nutritional value. Chemically pure samples of these compounds not being obtainable, an attempt was made to determine the lowest effective concentration of each of these compounds and of pure cholesterol. Only if this concentration proved to be considerably lower in the case of cholesterol than in that of the other compounds could the presence of small quantities of cholesterol as an impurity possibly give them an apparent positive nutritional value. It was

also considered of interest to ascertain whether the presence of such large quantities of sterols as had hitherto been used was essential or whether the quantities required might be more of the order of vitamin requirements.

Accordingly, series of diets with decreasing quantities of sterols were set up (Table 7). The threshold of 7-dehydrocholesterol was found to be considerably lower than that of cholesterol. Decreasing the quantities of 7-dehydrocholesterol from 10 to 0·5 mg./3 g. of food hardly altered the efficiency of the diet, so that the threshold must be considerably lower than this. With cholesterol at quantities below 2 mg. the efficiency decreased considerably, as seen by an increasing mortality and increasing time to pupation. Since, therefore, 7-dehydrocholesterol is more effective at low concentrations than cholesterol, the possibility that cholesterol as an impurity is the cause of the positive effect given by 7-dehydrocholesterol is ruled out. The same consideration applies to 7-dehydrocholesteryl monobenzoate, the threshold of action of which does not differ greatly from that of cholesterol.

Table 7. *Yeast fructose diet; 25°*

No.	Additions to diet mg./3 g. food	No. of larvae	No. pupating	First pupa at day	Last pupa at day	Average time of pupation days
31 (=16)	Cholesterol, 50	10	10	33	39	36
32	„ 10	10	10	31	41	36·5
		10	10	35	42	36·7
33	„ 2	10	10	33	41	37·9
		10	10	35	41	37·3
34	„ 1	10	6	35	45	40
35	„ 0·5	10	2	39	49	44
36 (=17)	Dehydrocholesterol, 10	10	10	33	38	35·5
37	„ 2	10	10	33	42	37·5
38	„ 1	10	9	33	41	37
39	„ 0·5	10	9	33	41	37
40	Dehydrocholesteryl monobenzoate, 5	10	10	34	45	38
41	„ 2	10	10	38	46	41
42	„ 1	10	9	38	57	47·8
43	„ 0·5	10	4	45	57	50·2

The experiments of Table 7 are, of course, not real threshold experiments, because the quantity of the substance which the insects have actually eaten during development is unknown. The amount of dehydrocholesterol in the food was not further decreased, since it was not believed possible to mix such small amounts evenly into the food.

Discussion

The only previous attempt to compare the roles of several sterols as food factors was undertaken by Van t'Hoog [1936] on *Drosophila*. *Drosophila* larvae were found to grow well on a sterile diet consisting of peptone from purified casein, sugar, a collodion dialysate of yeast extract, salts, agar-agar and water, if to this food was added a small quantity of the unsaponifiable fraction of fat [Van t'Hoog, 1935]. Later, Van t'Hoog [1936] found that this unsaponifiable fraction could be replaced successfully by a number of sterols: cholesterol (purified via the dibromide), sitosterol, stigmasterol, ergosterol, cholestanol (dihydrocholesterol) and cholestenone. Calciferol, on the contrary, and several derivatives of cholesterol, proved to be ineffective. The flour beetle *Tribolium confusum* also requires in its diet either cholesterol or ergosterol [Fröbrich, 1939; Offhaus, 1939]. The differences in the sterol requirements of two different insects, *Drosophila* and *Dermestes*, are remarkable. Cholesterol, sitosterol and stigmasterol

possess the same ring system with only one double bond in ring B and differ solely in the side chain on carbon atom 17. This difference is of no importance for *Drosophila* and of great importance for *Dermestes*, which cannot develop on sitosterol. Ergosterol and 7-dehydrocholesterol contain a second double bond in ring B and differ from each other only in the side chain. While ergosterol is active for *Drosophila* and *Tribolium* it is entirely inactive for *Dermestes*. This negative effect on *Dermestes* is obviously due to the side chain and not to the second double bond, because 7-dehydrocholesterol, which possesses the ring system of ergosterol and the side chain of cholesterol, is active for *Dermestes*. Cholestanol, which differs from cholesterol only in the absence of the double bond in ring B, is positive for *Drosophila* and negative for *Dermestes*, which indicates that the presence and position of the double bond is of importance too. Zymosterol, with one double bond in a different place in ring C and a side chain which is different from that of cholesterol, is also inactive.

Of the derivatives of cholesterol and dehydrocholesterol tested, only the esters of these compounds, cholesteryl acetate and 7-dehydrocholesteryl monobenzoate, have a positive effect, which suggests that hydrolysis of these esters takes place in the larva. Other changes in the configuration, such as the loss of the double bond in cholestanol, loss of the secondary alcohol group in Δ^5-cholestene, introduction of a second alcohol group in 7-hydroxycholesterol and esterification of this group in 7-hydroxycholesteryl dibenzoate or introduction of a keto-group on carbon atom 7 in 7-ketocholesteryl acetate, cause loss of nutritional value. It therefore appears, as far as can be judged from the number of compounds available for testing, that only substances with the configuration of cholesterol and dehydrocholesterol or their esters can be utilized by *Dermestes*. Change in the side chain, the number and position of double bonds, opening out of one ring, introduction of a second alcohol or a keto-group destroy the effect entirely.

The differences in the requirements for sterols in *Drosophila* and *Dermestes* are interesting. *Dermestes* feeds exclusively on relatively dry products of animal origin like stored hides, bacon, smoked meat, dried-up cadavers and the like in which cholesterol constitutes the only or main sterol present. *Drosophila* develops on moist food rich in carbohydrates in which yeast grows abundantly. In fact, it is generally considered that living yeast is the main food of the *Drosophila* larva [Guyénot, 1917]. It is therefore illuminating to find that the sterol requirements of *Drosophila* are fulfilled by ergosterol, the principal sterol of yeast, whereas as far as we know to-day cholesterol and 7-dehydrocholesterol, which appears to be generally associated with cholesterol, constitute the only active sterols for *Dermestes*. It will be shown in a later publication that the requirements of several flour insects for sterols are, amongst other sterols, met by sitosterol, the principal sterol of wheat germ oil.

In recent years the antirachitic effect of irradiated cholesterol on rats has been shown to be due to the presence before irradiation of 7-dehydrocholesterol as an impurity. It is, however, certain that for both *Drosophila* and *Dermestes* the presence of this impurity is immaterial, for purified cholesterol proved as effective as commercial cholesterol. The insects, then, require in their food a sterol, and it is not surprising that this requirement is not fulfilled by calciferol, in which the tetracyclic arrangement of the carbon atoms has been changed, and which is consequently not a true sterol.

So far it has been shown for at least five different insects, namely two flies, *Lucilia* [Hobson, 1935] and *Drosophila* [Van t'Hoog, 1936], three beetles, *Acanthoscelides* [Chiu & McCay, 1939], *Tribolium* [Fröbrich, 1939; Offhaus, 1939]

and *Dermestes*, and for a snail *Helix pomatia* [Howes, 1937], that the inclusion of a sterol in the diet is essential for growth. This also applies to four more beetles: *Lasioderma*, *Sitodrepa*, *Silvanus* and *Ptinus*, and one moth *Ephestia* [Fraenkel, unpublished]. It seems, however, that the requirements of living organisms for sterols are of a more general occurrence than has hitherto been supposed. Cailleau [1938] has shown that the parasitic flagellates *Trichomonas columbae* and *Tr. foetus* only grow in the presence of cholesterol. A certain strain (*W*) of yeast requires for growth a sterol in addition to bios; cholesterol is inactive but sitosterol, ergosterol and calciferol are active [Devloo, 1938]. The position therefore is that invertebrates and even some micro-organisms require sterols but do not require the irradiated products of sterols (vitamin D). In the light of these findings it seems that the almost universally accepted thesis of the synthesis of cholesterol in the mammalian body might require revision. Recent evidence on this subject is conflicting [Channon & Tristram, 1937; Rittenberg & Schoenheimer, 1937], and it has only recently been pointed out by Bacharach & Drummond [1940] that the question whether mammals are able to synthesize cholesterol is entirely unsettled.

Summary

1. Several methods for investigating the sterol requirements of the beetle *Dermestes vulpinus* have been described.

2. The larva of *D. vulpinus* only grows if the food contains a sterol. Active sterols include purified cholesterol (free from 7-dehydrocholesterol), cholesteryl acetate, 7-dehydrocholesterol and 7-dehydrocholesteryl monobenzoate. Sitosterol, ergosterol, zymosterol, cholestanol, cholestene, 7-ketocholesteryl acetate, 7-hydroxycholesterol, 7-hydroxycholesteryl dibenzoate and calciferol (vitamin D_2) are inactive.

3. The minimum quantity of sterol required for successful growth is about 1 mg./3 g. of food for cholesterol and 7-dehydrocholesteryl monobenzoate and less than 0·5 mg./3 g. of food for 7-dehydrocholesterol.

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76. THE USE OF MICRO-ORGANISMS IN SUGAR ANALYSIS

3. THE EFFECTS OF VARIOUS SALTS ON THE ACTIVITY OF *PROTEUS VULGARIS* IN REMOVING GLUCOSE AND SOME POSSIBLE SOURCES OF ERROR IN ITS USE AS A REAGENT FOR THE ESTIMATION OF GLUCOSE

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In the eight years since *Proteus vulgaris* was described as a reagent for the quantitative estimation of small amounts of glucose [Harding & Nicholson, 1933], continued experience has revealed a number of possible sources of error, and improvements in the methods of growth and use of the organism have simplified the procedure.

The effect of salt concentration

It was noted previously [Harding *et al.* 1936] that a concentration of 5% Na_2SO_4 inhibited completely the glucose-removing action of *P. vulgaris* and that a concentration of 3% led to a definite decrease in activity. The effects of varying concentrations of different salts which might be encountered in filtrates or hydrolysates of biological materials were determined. The results are shown in Table 1.

Table 1. *The effect of salt concentration on the glucose-removing power of* P. vulgaris

	% removal*					
Molar concentration	Na_2SO_4	K_2SO_4	NaCl	KCl	K_2HPO_4-KH_2PO_4†	Na_2HPO_4-NaH_2PO_4†
0·00	100	100	100	100	100	100
0·0075	—	—	—	—	350	—
0·015	112	130	119	142	350	231
0·02	110	128	123	158	—	—
0·03	110	124	132	188	350	231
0·05	86	119	140	184	—	—
0·07	70	104	135	180	350	230
0·15	43	63	103	148	350	164
0·20	30	52	85	114	—	74
0·25	24	43	73	89	350	21
0·35	0	32	53	63	—	—
0·45	—	31	34	55	—	—
0·55	—	31	23	50	—	—
0·65	—	30	17	46	—	—
0·75	—	29	—	—	—	—
1·50	—	—	16	44	350	20

* The amount of *P. vulgaris* used was just sufficient to remove 20% of the glucose from 10 ml. of a 20 mg. per 100 ml. solution in 30 min. at 37°. This removal was taken as 100 for purposes of comparison.

† These mixtures were made up so that the concentrations were of 0·918 *M* of the dibasic salt and 0·588 *M* of the monobasic salt in the stock solution. The *p*H of the various dilutions ranged from 6·9 in the strongest to 7·1 in the weakest solution.

Small concentrations of all the salts tested had an accelerating effect on the action of *P. vulgaris*. Potassium salts had a greater effect than sodium salts. Chlorides were more active than sulphates. At higher concentrations there is a marked inhibition of the removal activity, complete in the case of sodium sulphate at a concentration of 0·35 *M* and reaching a fairly constant level for each of the other salts tested: 30 % removal at concentrations of 0·35 *M* and over for K_2SO_4: 16 % removal at concentrations of 0·65 *M* and over for NaCl: 46 % removal at the same concentration or over for KCl. The sodium phosphate mixtures had a very similar effect, except that the activating effect of the lower concentrations was more marked. Potassium phosphate mixtures acted quite differently in that the activation was much more marked and was present throughout the range of concentrations employed.

The inhibiting effect of the higher salt concentrations has also been observed with *Gaffkya tetragena*, an organism which is used as a reagent for the removal of glucose and fructose [Nicholson, 1936], but the lower concentrations produce no increase in activity. This applies to the phosphates as well as to the other salts. No change whatever in sugar-removing power has been observed in the case of yeasts.

The marked activating power of potassium phosphate is not due in more than a minor degree to any buffering action, as is shown in Table 2.

Table 2. *Effects of phosphate, bicarbonate-carbonic acid and hydroxymethylglyoxaline hydrochloride-NaOH buffers on the activity of* P. vulgaris *in removing glucose from a solution containing 2 mg. glucose*

	No salt	KH_2PO_4-K_2HPO_4	$NaHCO_3$-*H_2CO_3	KCl	4 (or 5)-hydroxymethylglyoxaline hydrochloride + NaOH†
	—	0·03 *M*	0·03 *M*	0·03 *M*	[Kirby & Neuberger, 1938]
mg. glucose removed	0·28	1·0	0·56	0·43	0·53

All fermentations were carried out at a *p*H of 7·0. Removal in mg. from 10 ml. of a 10 mg. per 100 ml. solution of glucose using 0·10 g. *P. vulgaris* and incubating for one half-hour at 37–38°.

* A slow stream of CO_2 was run through the solution during incubation.

† We are indebted to Dr H. O. L. Fischer of the Department of Chemistry of this University for the dihydroxyacetone from which the hydroxymethylglyoxaline hydrochloride was prepared.

The glucose-removing activity of *P. vulgaris* may be further increased by adding potassium phosphate to the media in which the organisms are grown. The following media have been used:

Phosphate broth: 4375 ml. tap water, 625 ml. phosphate solution (see below), 50 g. NaCl, 30 g. glucose, 100 g. bacto-peptone (Lemco 62). Sterilize and tube in the usual manner.

Phosphate agar: 30 g. agar, 875 ml. broth, 50 ml. phosphate solution (see below), 10 g. glucose. Sterilize and bottle.

The potassium phosphate may be most conveniently added during the course of the dilution of the solution to be analysed for glucose. If, however, a stock 1·5 *M* phosphate mixture (16 g. K_2HPO_4 + 8 g. KH_2PO_4 in 100 ml. water) is used, the dilution (0·5 %) involved in adding the potassium phosphate to the actual solution being fermented is negligible.

The batches of *P. vulgaris* grown on such media showed 60 % greater activity than did those grown on the media originally described [Harding & Nicholson, 1933]. When *P. vulgaris* grown on phosphate-containing media is used on glucose solutions containing potassium phosphate (0·0075 *M*), the glucose-removing power of the organisms is so increased that 0·5 g. of wet-

weight packed organisms will remove 2·5 mg. of sugar by incubation for 10 min. at 37°. When grown on the original media and acting without phosphate added to the glucose-containing solution it takes 0·5 g. wet-weight packed bacteria and incubation for half an hour at 37° to remove 1 mg. of glucose. These procedures have no effect on the specificity of *P. vulgaris* for glucose.

The accelerating action of phosphates on yeast juices and maceration extracts is well known, as is the effect of higher concentrations in yeast-juice fermentation in delaying the rate of CO_2 production [Harden & Young, 1908; Harden, 1932]. Meyerhof [1918] noted the retarding effect of salts other than phosphates and Harden & Henley [1921] observed that sulphates had a greater retarding effect than chlorides. In the case of *Proteus*, however, the accelerating effect of phosphate is noted in the intact organism, not in its extracts only as in the case of yeast. Most of the observations on the retardation effects of salts on yeast have been made on extracts, but Lampe & Kilk [1931] noted that the NaCl formed by the neutralization of HCl used in the inversion of cane sugar decreased the alcohol yield on fermentation. The action of *P. vulgaris*, however, is retarded more by salts of sodium than by those of potassium, whereas yeast extracts are affected equally by either [Harden & Henley, 1921]. In the case of *P. vulgaris* there is also an accelerating effect by small concentrations of chlorides and sulphates as well as phosphates and the potassium phosphate has no retarding effect even in relatively high concentrations.

The effect of p*H*

It was previously reported [Harding & Nicholson, 1933] that *P. vulgaris* would not act on glucose at a *p*H lower than 6·5 or higher than 8·0. It has been found, however, that it is inadvisable to use this micro-organism at any *p*H higher than 7·2 because, although the bacteria are still active in removing glucose in the more alkaline solutions, there is a small amount of lysis with the liberation of varying amounts of reducing substances. If *Proteus* alone is being used this does not affect the accuracy of the glucose estimation, provided that the blank is incubated at the same *p*H as the solution being tested, but if a differential fermentation is performed it is essential that the reaction be kept below *p*H 7·2, preferably between *p*H 6·5 and 7·0 because some of the products of the bacteriolysis are acted on by yeasts to give reducing products. This action is illustrated in Table 3.

Table 3. *Action of* P. vulgaris *followed by* Monilia Krusei *on a solution containing 4 mg./100 ml. glucose and 4 mg./100 ml. fructose* (a) *in neutral solution* (p*H 7·0*), (b) *in alkaline solution* (p*H 7·8*)

	P. vulgaris			*P. vulgaris* followed by *M. Krusei*		
Total reduction	Blank	Reduction after fermentation	Removal	Blank	Reduction after fermentation	Removal
(*a*) 0·69	0·00	0·34	0·35	0·02	0·02	0·34
(*b*) 0·69	0·09	0·43	0·34	0·17	0·72	Added 0·21

The reducing values were determined using the Harding & Downs [1933] reagent. 1 ml. of reagent and 1 ml. of sugar solution were used. The results are expressed as ml. of 0·005 *N* $Na_2S_2O_3$.

This phenomenon is of particular importance in solutions containing any considerable amount of urea, because *P. vulgaris* will produce sufficient ammonia from the urea to make the solutions quite alkaline. Such solutions must therefore be well buffered before the introduction of *P. vulgaris*, or preferably be freed from urea.

The effect of the presence of nitrates

P. vulgaris converts nitrates into nitrites. The nitrites will liberate I_2 from KI in acid solution and therefore interfere with any iodimetric sugar estimation. The sugars may be obtained free from nitrates and other interfering substances by the modified copper-lime technique [Harding *et al.* 1936], or the nitrites may be converted into NH_3 by saturating the solution with H_2S and shaking vigorously in presence of air for a few minutes. The H_2S is then removed by aeration; the NH_3 is removed by the use of KH_2PO_4 and MgO [Harding & Downs, 1933].

Some modifications in the methods of using P. vulgaris

The great enhancement of the glucose-removing power of *P. vulgaris* in the presence of potassium phosphate together with the use of Lloyd's reagent for final clearing of the *P. vulgaris* from the fermented solutions after centrifuging [Griffiths & Waters, 1936] has led to the adoption of the following procedure. The organisms are washed off the phosphate agar surface, packed in the angle centrifuge, then rewashed once in distilled water. A 25 % suspension of the wet packed bacteria is made in distilled water. 2 ml. of the suspension (containing 0·5 g. *P. vulgaris*) are taken for each 2·0 mg. of glucose to be removed and the organisms are packed and dried as described by Harding & Nicholson [1933]. Add to each 10 ml. of the sugar solution to be tested, preferably during dilution, 0·05 ml. of a solution of potassium phosphate:

$$\left.\begin{matrix} K_2HPO_4\text{—16 g.} \\ KH_2PO_4\text{—8 g.} \end{matrix}\right\} \text{ in 100 ml. } H_2O.$$

Incubate in the water bath at 37–38° for 10 min. and centrifuge. Clear the few remaining organisms from the supernatant fluid by shaking with a little Lloyd's reagent and filtering through a fluted filter paper [Griffiths & Waters, 1936].

If no angle centrifuge is available the following procedure may be followed. Wash the bacteria from agar, place not more than 7 ml. of the suspension in a 15 ml. conical centrifuge tube and centrifuge at 2500 revolutions per min. for 15 min. Pour off the cloudy supernatant fluid. Add 5 ml. of distilled water, resuspend and recentrifuge. Pour off cloudy supernatant and recentrifuge for 15 min. Make up a 50 % suspension of the bacteria. Put not more than 0·5 ml. of the suspension (sufficient to remove 1 mg. glucose in 10 min.) in a 15 ml. centrifuge tube, centrifuge for 15 min., pour off the supernatant fluid and recentrifuge for 5 min. Pour off any fluid remaining and dry the sides of the centrifuge tube. Incubate with the solution to be tested (containing not more than 1 mg. glucose) in the presence of 0·0075 *M* phosphate. After incubation centrifuge for 15 min. Clear the markedly cloudy supernatant fluid by shaking with 1·2 g. Lloyd's reagent and filtering.

SUMMARY

The ability of *P. vulgaris* to remove glucose from solution is enhanced by small concentrations of NaCl, Na_2SO_4, KCl and K_2SO_4 and is greatly inhibited by larger concentrations.

The potassium salts are the more active accelerators and sodium salts are the stronger inhibitors. Sulphates show a greater inhibiting effect than do chlorides.

Sodium phosphate in small concentrations and potassium phosphate in all concentrations greatly enhance the glucose-removing ability of the organisms, potassium phosphate increasing the glucose removal 3·5 times.

Fermentation in a solution more alkaline than *p*H 7·2 introduces errors due to the dissolving of reducing substances from the cells.

The reduction of nitrates to nitrites by *P. vulgaris* may lead to errors in iodimetric titrations. Methods for obviating this difficulty are described.

A method for using *P. vulgaris* without an angle centrifuge is given.

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77. CORRELATION BETWEEN SEX HORMONES, THYROID HORMONES AND DESOXYCORTICOSTERONE AS JUDGED BY THEIR EFFECTS ON THE WEIGHTS OF ORGANS OF GONADECTOMIZED RATS

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That there is a correlation between the endocrine secretions of the adrenal, thyroid and sex glands has repeatedly been suggested in the literature on indirect evidence [see survey of literature, Cramer, 1928; Harington, 1933; Grollman, 1936; Means, 1937; Crotti, 1938]. At the same time, the adrenal, thyroid and sex glands are considered to play some part in a manifold mechanism responsible for the maintenance of physical and mental vigour of the organism.

Therefore an investigation of the interrelation of the hormones secreted by these glands is desirable not only for theoretical reasons, but also for possible practical purposes, since these hormones are already extensively used, although not always on sufficiently scientific grounds, in the treatment of different pathological conditions.

The effect of these hormones, administered separately, on the weights of some organs has been investigated extensively in the case of sex and thyroid hormones, but very little in the case of desoxycorticosterone. In order to avoid repetition, a brief survey of this literature will be given for each hormone in the paragraphs dealing with the respective hormones. As far as we know, the correlative changes in weight and structure of organs produced by these three groups of hormones, sex, thyroid, and corticoadrenal, when administered simultaneously, have not been studied.

In order to elucidate this problem, we undertook a number of experiments, some of which are still in progress. In this paper we shall include only some of the results of the first series of experiments, namely, changes in the weights of the organs. The results of their histological examination will be published elsewhere.

Technique

For the experiments 168 rats were used, 86 males and 82 females. Ten rats were kept as normal controls, and all the remaining animals were gonadectomized when 22–24 days old. The final average age of the rats varied from 167 to 207 days. The distribution of the rats into the different groups, their number in each group, the final body weight and the weights of those organs which were changed in at least some of the groups are given in Tables 1 and 2. All animals were killed by bleeding.

[1] Dr Korenchevsky desires to express to Oxford University, to Prof. E. S. Goodrich and to Prof. J. A. Gunn his own and the Lister Institute's gratitude for the kind hospitality extended to him and his co-workers in the Department of Zoology and Comparative Anatomy and the Nuffield Institute for Medical Research.

Table 1. *The effects of male, oestrogenic and thyroid hormones and desoxycorticosterone, administered alone or in combination,*

Table 1. *The effects of male, oestrogenic and thyroid hormones and desoxycorticosterone, administered alone or in combination, on actual weights of organs of castrated* rats

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV
			Castrated rats treated with hormones												
					Testosterone propionate			Androsterone			Desoxycorticosterone acetate			Oestradiol B.B. 0·09 mg.	
	Control rats		Thyroid hormones			+Thyroid hormones			+Thyroid hormones			+Thyroid hormones			+Thyroid hormones
	Normal	Castrated	Small dose	Large dose	Alone	Small dose	Large dose	Alone	Small dose	Large dose	Alone	Small dose	Large dose	Alone	small dose
Seminal vesicles (mg.)	1049	13	13	15	1298	1299	1052	61	38	51	14	12	17	80	96
Prostate (mg.)	1394	60	61	67	1382	1133	1217	188	169	185	62	72	62	105	122
Penis (mg.)	357	84	80	70	348	353	336	183	157	150	75	91	63	79	77
Preputial glands (mg.)	95	30	34	31	108	114	99	66	68	73	30	36	38	40	42
Adrenals (mg.)	54	58	58	93	50	49	74	47	47	70	49	53	87	70	88
Hypophysis (mg.)	13·1	15·9	14·2	15·1	14·6	13·4	13·6	13·2	14·4	14·3	16·1	18·3	15·4	41·5	47·7
Thymus (mg.)	167	224	222	111	96	114	117	185	220	118	232	266	122	132	156
Liver (g.)	11·28	8·65	8·36	10·58	10·24	10·17	10·63	9·55	9·73	9·87	9·44	11·73	10·90	8·76	11·09
Kidneys (g.)	2·24	1·66	1·61	2·37	2·03	2·01	2·79	1·83	1·91	2·34	1·90	2·27	2·87	1·82	2·47
Spleen (mg.)	645	848	807	690	738	836	739	687	889	658	702	1324	709	733	885
Heart (mg.)	1101	943	982	1398	1084	1126	1419	1043	1118	1290	999	1270	1437	901	1070
Retroperitoneal fat (g.)	7·3	9·5	8·3	1·7	7·1	9·6	2·2	11·8	10·9	1·1	10·8	11·3	1·1	11·1	7·7
Final body weight (g.)	334	293	272	258	313	319	287	324	313	248	306	350	254	257	277
No. of rats in group	4	12	8	3	9	7	3	7	6	3	7	5	2	5	5

Note. In both tables, all the 'small dose' groups belong to the first experiment, the 'large dose' groups to the second experiment, and they should be compared accordingly.

Table 2. *The effects of male, oestrogenic and thyroid hormones, administered alone or in combination, on actual weights of organs of ovariectomized rats*

	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII	XIII
			Ovariectomized rats treated with hormones										
								Oestradiol B.B. 0·015 mg.			Oestradiol B.B. 0·015 mg.		
					Androsterone								
	Control rats		Thyroid hormones			+Thyroid hormones			+Thyroid hormones	+Androsterone+thyroid hormones		+Thyroid hormones	+Androsterone+thyroid hormones
	Normal	Ovariectomized	Small dose	Large dose	Alone	Small dose	Large dose	Alone	small dose	small dose	Alone	large dose	large dose
Uterus (mg.)	409	31	39	31	36	40	28	191	220	240	207	238	368
Vagina (mg.)	292	160	187	178	236	224	234	273	304	333	255	307	365
Preputial glands (mg.)	74	49	53	52	131	116	161	48	44	123	49	49	212
Adrenals (mg.)	63	62	63	103	51	52	85	55	84	77	98	166	149
Hypophysis (mg.)	15·1	14·5	14·7	13·4	16·1	13·8	14·5	13·8	15·8	15·4	20·2	20·5	18·8
Thymus (mg.)	136	184	228	192	194	170	137	125	158	133	167	176	152
Liver (g.)	6·66	7·22	7·94	9·53	9·08	9·44	10·75	5·97	7·27	8·21	9·03	13·20	11·70
Kidneys (g.)	1·58	1·53	1·67	2·41	1·75	1·81	2·44	1·34	1·69	1·84	1·79	2·98	3·05
Spleen (mg.)	479	838	788	838	595	783	1564	460	797	978	581	1053	906
Heart (g.)	795	828	953	1158	941	1055	1345	783	907	984	921	1383	1247
Abdominal fat (g.)	17·3	10·4	12·4	0·5	12·8	15·7	1·0	12·0	9·8	17·0	9·0	1·0	2·3
Final body weight (g.)	218	256	262	213	289	288	247	220	223	262	227	233	247
No. of rats in group	6	11	10	3	9	6	3	5	5	6	3	3	3

Only those hormones which are naturally present in the organism were used (administered, however, mostly as esters). The following hormones were given, in the doses indicated per week: in experiments on males—androsterone 4·5 mg., testosterone propionate 0·750 mg. and oestradiol 3-benzoate-17*n*-butyrate 0·09 mg. (abbreviated in the paper to 'oestradiol B.B.'); in experiments on females—androsterone 4·5 mg., oestradiol B.B. 0·015 mg. In one experiment (Table 2, cols. VIII–X) this oestrogen was injected only for the first 3 weeks; in the second experiment (cols. XI–XIII) for the first 3 weeks, and also during 2 weeks towards the end of the experiment.

Thyroxine, thyroid powder and desoxycorticosterone were administered to both sexes; the thyroid powder was given by pipette in gum acacia suspension sweetened with honey. The groups receiving a 'small dose' of thyroid hormones were given 30–40 mg. of thyroid powder (Parke, Davis and Co.) three times weekly; the duration of this experiment was 54–58 days. The groups receiving the 'large dose' were fed with 40 mg. three times weekly for the first 3 weeks, 80 mg. three times weekly for the next 6 weeks, and 80 mg. five or six times weekly for the last 2 weeks; this group also received during the last 3 weeks of the experiment 1 mg. injected subcutaneously one to three times weekly (total 9 mg. per rat) of thyroxine sodium (British Drug Houses). The variation in the dose of thyroid hormones was dependent upon the loss in weight and appetite, in order not to impair too much the general condition of the rats. The duration of the 'large dose' experiment was 76 days.

The rats received desoxycorticosterone in the form of two subcutaneously implanted crystalline pellets, one of acetate (50 mg.) and the other of pure hormone (25 mg.). The group of rats receiving the large dose of thyroid received also in addition to the two implanted pellets of desoxycorticosterone, subcutaneous injections of 2–4 mg. weekly of desoxycorticosterone acetate in oil. No difference was observed in the weights of the organs of these rats as compared with those of rats receiving desoxycorticosterone in the form of pellets only. Therefore in Table 1, the data of these two groups have been pooled.

For the same reason the data from the two experiments have been pooled in the tables in the case of control rats or rats injected with testosterone propionate alone.

In other respects the conditions of the experiments were the same as those described in our previous papers. The diet was similar to that previously described [Hall & Korenchevsky, 1938] except that fish was not always obtainable, and the yeast used, although obtained from the same source, was poorer in B-vitamins.

The weights of the organs were also calculated per unit of body weight, but for economy of space the results are not tabulated here. When of importance, they will be discussed in the text.

Effect of gonadectomy and of sex hormones

In both males and females the effects of gonadectomy (Tables 1 and 2, col. II), male sex hormones (Table 1, cols. V and VIII; Table 2, col. V) and oestrogens (Table 1, col. XIV; Table 2, cols. VIII and XI) were similar to those described in our previous papers [1933–41].

Thus, the *body weight* of castrated males was less, and of ovariectomized females was greater than that (col. I) of the respective normal controls. In both sexes the male hormones, in the comparatively small doses used, caused a slight increase in the body weight when compared with gonadectomized controls. Oestrogens, on the other hand, decreased the gain in body weight, as has been

noted several times in our own papers and in those of other workers. That there was only a slight depression of growth in the present experiments is explained by the use of the butyrate-benzoate ester of oestradiol, the effect of which is mild if sufficiently long intervals (in this case, at least 1 week) are allowed between the injections.

Fat deposition. The same explanation applies to the absence of any pronounced depression of fat deposition by oestradiol benzoate-butyrate (Table 2, col. XI), or even to a slight increase (Table 1, col. XIV; Table 2, col. VIII). Usually, as our previous experiments have shown, oestrogens depress fat deposition. In both sexes, androsterone caused a slight increase and testosterone propionate a slight decrease in the amount of fat deposited.

In our earlier papers we have shown that weak male hormones such as androsterone, or small doses of strong hormones cause no change or a slight increase in the amount of fat deposited; while medium doses and, especially, large doses of strong male hormones decrease the amount of adipose tissue in the organism. As has been emphasized previously [e.g. Korenchevsky, Dennison & Eldridge, 1937, 1, p. 469; 1937, 2, p. 478; Korenchevsky, Dennison & Hall, 1937, 1, p. 782; Korenchevsky, Hall & Ross, 1939, p. 214, Table 2, col. XIII) it often happens that after the administration of male hormones, while (as compared with the gonadectomized controls) fat deposition is decreased, the body weight remains unchanged or is even increased. In the present experiments this occurred in the case of testosterone propionate (Table 1, col. V). These observations appear to be important, since they suggest that the sex hormones may have anabolic properties. Thus, the increased gain in body weight is probably to be explained by an increase in the anabolic processes of building new tissues (e.g. in liver, kidneys, heart, among others) and not by a passive accumulation of fat in the organism. Instances indicating catabolic properties of large doses of oestrogens and male hormones have been given in our previous papers.

Sex organs. The usual return to or towards normal was observed, the degree of change depending upon the strength of the compound and the size of the dose. The specific effect of oestrogens on male sex organs has been fully discussed and the literature reviewed in our previous papers [e.g. Korenchevsky, 1941]. We wish to emphasize here only one detail of the effect of androsterone (a hormone naturally present in females) on the female sex organs, in confirmation of our previous observation [Korenchevsky, Hall & Burbank, 1939]. Lengthening the period of injection of this hormone increases the hypertrophy of the vagina and female preputial glands, but not of the uterus (Table 2, col. V).

Adrenals. The literature has been reviewed by Hall & Korenchevsky [1938] and Hall [1940]. In the present experiments, no effect of gonadectomy was noticed, but as before male hormones caused a decrease in weight and size of the glands, the mild effect being due to the comparatively small doses used. As before, the effect of oestrogen was not constant, but in those groups in which it was injected for a longer period (Table 2, col. XI) or in a larger dose for a long period (Table 1, col. XIV) the adrenals were definitely enlarged. Histological observations [Hall, 1940] suggest a possible explanation of these variations. Hyperaemia in the adrenals of animals treated with oestrogens varies in degree, in some cases being very pronounced. Our rats were killed by bleeding. The varying degree of hyperaemia, and the emptying of the hyperaemic areas by bleeding would have a considerable effect upon the weight of the organ.

The hypophysis was hypertrophied after prolonged injections of small doses (Table 2, col. XI) and large doses (Table 1, col. XIV) of oestrogens. In males

there was a slight 'castration' hypertrophy of the gland (Table 1, col. II) which was restored to normal by injections of androsterone (col. VIII). All these data are in agreement with our previous results [e.g. Korenchevsky, Hall & Ross, 1939].

Thymus. The literature has been reviewed and the results of our previous experiments summarised by Ross & Korenchevsky [1941]. The thymus, which is always enlarged after gonadectomy, decreases in size and weight after injections of male hormones and oestrogens. In the present experiments the only exception to this rule was the absence of the usual response of the thymus to injections of androsterone in females (Table 2, col. V). This sometimes happens after treatment with weak hormones or small doses of strong hormones.

Liver, kidneys and heart. Again our present data confirm our previous results, showing hepato-, nephro- and cardio-trophic properties of male hormones. Thus a slight atrophy of these organs is obtained after castration in males (Table 1, col. II) and a return towards normal after injections of male hormones (Table 1, cols. V and VIII) but not of oestrogens. Gonadectomy (as also in these experiments) does not usually have an atrophying effect on these organs in females. Still, the hepato-, nephro- and cardio-trophic properties of male hormones are clearly demonstrated also by the effects of the injections into gonadectomized females in which the liver, kidneys and heart become larger than in the intact or gonadectomized uninjected controls (compare Table 2, col. V with cols. I and II).

In rats of the same breed, kept under the same conditions and on the same diet, the liver, kidneys and heart are larger in males than in females. Therefore in our previous paper we defined the action of male hormones on the liver, kidneys and heart of females as a masculinizing effect [Korenchevsky, Hall Burbank & Cohen, 1941, p. 398].

As before, the effect of oestrogens on the actual weights of these organs was not constant, but a regular enlargement of liver, kidneys and heart is always shown in the relative weights. If, however, such mild and less toxic oestradiol esters as the benzoate-butyrate are used with sufficiently long intervals between the injections, it is sometimes possible to obtain hypertrophy of these organs even in actual weight, as happened, for instance, in one of the present experiments (Table 2, col. XI). These facts suggest that under certain conditions, and in the right doses, oestrogens might also possess non-sexual stimulating properties similar to those of male hormones.

All the data in our previous papers [1933–9] demonstrating the hepato-, nephro- and cardio-trophic properties of sex hormones have recently been summarized and examined in detail [Korenchevsky & Ross, 1940; Korenchevsky, Hall, Burbank & Cohen, 1941]. The nephrotrophic property of testosterone propionate has also been investigated and confirmed by Selye [1939; 1940], Ludden *et al.* [1941] and Kochakian [1941], the latter also confirming the hepato- and cardio-trophic effects.

Spleen. As found in our previous experiments, the effects of gonadectomy and of sex hormones on this organ are not constant, but in some of the experiments there were pronounced and definite changes. A detailed investigation of the data from all our experiments will be published elsewhere.

Effect of thyroid hormones

Cameron [1930] and Cameron & Carmichael [1920; 1921; 1922; 1924; 1926] give an account of the literature and describe their own numerous and important experiments.

The thyroid hormones administered by mouth in the form of dried thyroid powder produce hypertrophy of adrenals, liver, kidneys, heart and, less constantly, of spleen and lymphatic glands, loss of fat tissue, decrease of the growth rate or, with large doses, actual loss of body weight of animals. Four to seven weeks after thyroid feeding has been stopped [Cameron & Carmichael, 1922], the hypertrophied organs usually return to normal, while the animal's growth rate becomes accelerated above normal to such an extent that the weights of the treated rats usually exceed those of the controls.

Thyroxine, although found to possess every known activity of thyroid secretion, has not so far been proved with certainty to contain the maximum of thyroid activity. The best definition of the question is given by Harington [1933, p. 169]: 'it therefore seems unnecessary to assume the existence in the thyroid of any specifically active principle other than thyroxine, so long as we are careful to bear in mind that thyroxine is not to be regarded as itself the complete active principle, but is rather to be considered as the constituent which confers specific physiological properties upon a more complex molecule'. According to Cameron & Carmichael [1921] thyroxine administered by mouth produces hypertrophy of the same organs as in rats fed with thyroid powder. Sternheimer [1939], injecting thyroxine, confirmed the hypertrophy of the liver, and Uotila [1940] of adrenals. Uotila found no definite change in the weight of the testes, but a decrease of about 40 % in the weight of the seminal vesicles.

In the present experiments the small dose of thyroid hormones had practically no effect in males and very little in females (Tables 1 and 2, col. III). This 'small dose' is to be considered comparatively large for rats, and the lack of response to this dose in our experiments can probably be explained by the richness of our diet in vitamin A. This vitamin has been shown to be antagonistic to the thyroid hormones and therefore more or less neutralizes their effects (see the Survey of Literature and their own experiments, Smith & Perman [1940]). With the large dose we obtained the usual effects, thus confirming the results recorded by previous authors: the growth rate, final body weight and fat deposition were decreased; adrenals, liver, kidneys, heart and, not constantly, spleen were hypertrophied. No effect was found on the weight and size of the sex organs and hypophysis of gonadectomized rats (Tables 1 and 2, col. IV). The large dose produced a definite atrophying effect on the thymus only in males. When the weights of the hypertrophied organs of gonadectomized rats treated with the large dose of thyroid hormones are compared with those of normal untreated rats, it is found that they often considerably exceeded normal level, especially in females.

Effect of desoxycorticosterone

According to Selye [1940] and Ludden *et al.* [1941], desoxycorticosterone causes enlargement of the kidneys and [Hooker & Collins, 1940] has some stimulating activity on male sex organs.

In the present experiments (Table 1, col. XI) slight hypertrophy of liver and kidneys was found, but no definite effect was noticed on the other organs examined, or on fat deposition or body weight.

Similar small changes have been obtained in another experiment of 21 days' duration (not included in the Tables) on 11 young normal males (final age 52 days): the weights in the control rats were—liver 12·0 g., kidneys 2·3 g., heart 0·949 g.; and in the injected rats—liver 12·91 g., kidneys 2·53 g., heart 1·080 g.

Correlation between the male sex and thyroid hormones

Males. When castrated rats were treated simultaneously with both thyroid and sex hormones, the effect obtained (Table 1, cols. VI, VII, IX, and X) did not exceed that produced by each hormone separately in the case of thymus, liver, kidneys, spleen, heart and fat deposition. Small changes in the weights of sex organs and hypophysis are not conclusive, unless confirmed on a larger number of animals, or unless histological examination shows some effects.

A definite neutralizing or antagonistic effect was found on adrenals: the hypertrophy of this gland produced by large doses of thyroid hormones (weight of adrenals 93 mg.) was lessened by the addition of testosterone propionate (74 mg.) or androsterone (70 mg.). As has been emphasized in our previous papers, this action on adrenals is a typical property of male hormones.

Another effect of male hormones antagonistic to that of thyroid hormones was shown in the gain in body weight: when thyroid hormones were administered alone, the gain was 27 g. with the small dose and 7 g. with the large dose; when testosterone propionate was injected simultaneously, the respective figures were 76 and 32 g. The neutralizing effect of androsterone was considerably less pronounced, the gain being 38 and 10 g. respectively, i.e. androsterone was unable to neutralize the effect of the large dose of thyroid hormones. It should be emphasized, however, that two rats of this group suffered from slight pneumonia, and perhaps the experiments now in progress on a larger number of animals will give more definite results. This can reasonably be expected when one considers the results on females described below.

Females. The antagonistic action of androsterone on the effect of thyroid hormones on adrenals and gain in body weight of females (Table 2, cols. VI and VII) was similar to that of testosterone propionate in males. For example, the gain in body weight of females treated with thyroid hormones alone was 24 g. (small dose) and 5 g. (large dose), while with simultaneous injections of androsterone these figures were 39 and 20 g. respectively.

As in males, there were no correlative changes in the weights of the sex organs, hypophysis and fat deposition in females.

A smaller thymus and larger spleen in rats treated with androsterone and the large dose of thyroid must be interpreted with caution, since the number of animals used was small, and the weights of all lymphatic organs sometimes vary considerably.

In females, unlike males, liver, kidneys and heart were in most cases larger than in rats receiving either hormone separately. When calculated per unit of body weight, however, these differences are less definite, so that in the case of these organs a co-operative stimulating activity can be suggested only with reservation.

Correlation between oestrogens and thyroid hormones

In contrast to the poor correlation with male hormones, thyroid hormones have a manifold interrelation with oestrogens as shown by both actual (Table 1, col. XV; Table 2, cols. IX and XII) and relative weights of several organs. The organs affected are the same in both sexes. The degree of correlation, however, differs in different organs and with different doses.

A weak co-operative activity, leading to slightly increased hypertrophy of the organs, seems to be present in some of the sex organs, the seminal vesicles and prostate of males, and the uterus and vagina of females. There is, however, no stimulating effect on the penis of males, or the preputial glands of either sex.

Because of this weak co-operative activity on the sex organs, it will be necessary to confirm the conclusions by histological investigation.

Pronounced co-operative activity of thyroid hormones and oestrogens is shown in both sexes by greater hypertrophy of adrenals, liver, kidneys and heart in rats treated with both hormones as compared with those given either hormone separately (compare in Table 1, col. XV with cols. III and XIV and in Table 2, col. XII with cols. IV and XI); or by the return to normal of the liver, kidneys and heart, the development of which is depressed by oestradiol alone (compare in Table 2, col. IX with cols. III and VIII). The fact that there is a greater loss of fat, and a decrease in the growth rate or a greater loss of body weight when both hormones are given together than when each is given separately, can also be considered as a form of co-operative activity.

The weight of the thymus remains unchanged or is even slightly increased, while the spleen, which becomes smaller after oestrogen treatment, is enlarged when both hormones are given together.

Correlation between oestrogens, thyroid hormone and androsterone in females

Since these three groups of hormones are naturally present in the female organism, simultaneous administration of these compounds to gonadectomized females was undertaken, in one experiment using a small dose (Table 2, col. X) and in another experiment a large dose (col. XIII) of thyroid hormones.

In both experiments better development of the uterus and vagina, and in one experiment (col. XIII) of the preputial glands was obtained. It is noteworthy that this co-operative activity was considerably more pronounced when the larger dose of thyroid hormones was used (col. XIII) although thyroid hormones when injected alone (cols. III and IV) have no definite effect on the weight of the sex organs.

When the small dose of thyroid hormones was combined with androsterone and oestrogens, a slightly better development of liver and kidneys was obtained which, however, did not reach those of androsterone-injected (col. V) rats. As this was not shown by the relative weights of these organs, however, the enlargement must be ascribed at least in part to the greater body weight of the animals of this group.

As shown above, decrease of growth rate or loss of body weight and loss of fat tissue, and also hypertrophy of adrenals are considerably intensified when oestrogens and thyroid hormones are administered simultaneously. Androsterone, when given in combination with these two compounds, had only a weak neutralizing (antagonistic) effect, especially when the large dose of thyroid was used. In the case of adrenals, this neutralization was insignificant. As described above, however, the neutralizing effect of androsterone was considerable when it was given in combination with thyroid hormones alone. This indicates that, as with every regulating mechanism, there are limits beyond which the regulating adjustment becomes inefficient.

Correlation between desoxycorticosterone and thyroid hormones in males

As shown in Table 1, cols. XII and XIII, no definite correlative changes were noticed in the sex organs, adrenals, hypophysis and thymus of rats injected with these two hormones.

Some neutralizing effect of desoxycorticosterone, similar to that of male hormones, on the changes produced by small doses of thyroid was present in the

case of gain in body weight and fat deposition, which showed an improvement when compared with the effects of the small dose of thyroid hormones alone (Table 1, col. III). However, this neutralizing effect did not appear when the large dose of thyroid hormones was used in combination with desoxycorticosterone (col. XIII).

A definite co-operative activity, especially with the small dose of thyroid hormones, was present in the case of liver, kidneys, spleen and heart. These organs were considerably larger when both hormones were combined than when either was given separately.

Before summarizing our results, it must be emphasized that all our statements are valid only for the correlative changes in weight of the organs. Histological and chemical investigation and new experiments approaching the problem from a different angle will make clearer and more precise the interrelation of the hormones.

Summary

1. Ten intact normal and 176 gonadectomized male and female rats were used in order to investigate the possible correlation between the sex and thyroid hormones and desoxycorticosterone, as judged by changes in the weights of the organs.

2. The changes produced by gonadectomy and the injections of sex hormones described in our previous papers were confirmed, including hepato-, nephro- and cardio-trophic properties of male sex hormones and, possibly, of some oestrogenic compounds.

3. The effects of thyroid hormones found previously by other workers were also confirmed. Our large dose produced decreased gain in body weight, loss of fat tissue, hypertrophy of adrenals, liver, kidneys and heart.

4. The small dose of thyroid hormones used in our experiments, although producing only slight or no changes when administered alone, yet had definite and sometimes very pronounced effects, co-operative with or antagonistic to the other hormones in several instances.

5. Desoxycorticosterone when injected alone produced only slight hypertrophy of the kidneys (confirming the results of previous workers) and of the liver.

6. The co-operative activity of thyroid hormones with oestrogens was manifested in a slightly increased hypertrophy of some of the sex organs. In females this co-operative effect was enhanced when a third hormone naturally present in females, namely androsterone, was given simultaneously.

7. Another form of co-operative activity was shown by an increase of the hypertrophy produced by thyroid hormones in liver, kidneys and heart, when oestrogens (in both sexes), or desoxycorticosterone (in males) or possibly male hormones (in females) were administered simultaneously with the thyroid hormones.

8. Co-operative activity between oestrogens and thyroid hormones was also apparent in the increased size and weight of adrenals, slower growth rate or greater loss of body weight and greater loss of fat tissue when the two hormones were combined than when each was given alone.

9. Antagonistic (neutralizing) activity of male hormones appeared in a return towards normal level of the gain in body weight and fat deposition depressed by thyroid hormones alone or in combination with oestrogens. Testosterone propionate was more effective in this respect than androsterone.

10. Another effect of androsterone and testosterone propionate antagonistic to thyroid hormones was shown in some reduction of the hypertrophy produced

in the adrenals by thyroid hormones, but this neutralizing property of androsterone was apparently ineffective in reducing the greatest degree of adrenal hypertrophy produced by simultaneous injections of oestrogens and thyroid hormones.

Grants from the Medical Research Council and the Lister Institute have enabled us to carry out this work and to them our thanks are due. We are much indebted to Messrs Ciba Ltd., in particular to Dr K. Miescher, for the generous supply of the hormones, and also to Messrs Parke, Davis and Co. for the supply of thyroid powder.

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78. SERUM GONADOTROPHIN

I. PREPARATION OF A STABLE CONCENTRATE FROM PREGNANT MARE'S SERUM

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Several different methods have been proposed for the preparation of gonadotrophic concentrates from pregnant mares' serum, but the majority of these fail to achieve adequate concentration of activity in relation to total weight, without serious loss of active material. Goss & Cole [1931] employed sodium sulphate fractionation and reported a ten-fold concentration of activity in the pseudoglobulin fraction. Evans *et al.* [1933] proposed using aluminium hydroxide as an adsorbing agent and this was applied to fresh plasma by Gustus *et al.* [1936]. Catchpole & Lyons [1934] used acetone for fractionation but achieved little separation from the serum proteins, while Meyer's [1935] method, using butanol and acetone, proved troublesome owing to the interference of inorganic salts.

The method which has so far proved most acceptable is that of Cartland & Nelson [1937] with the modifications introduced by Goss & Cole [1940]. It depends upon fractional precipitation of the serum with acetone at different *p*H levels; very large volumes of solvent are required. Cartland & Nelson state that a product substantially free from serum proteins may be obtained, representing 65 % of the initial activity. Goss & Cole find that recovery after the first stage, even when improved according to their directions, does not average more than 60 %, and the overall yields of their final preparations are not stated, although high potencies are claimed.

The use of salicylsulphonic acid at *p*H 1·9–2·2 to remove the inert proteins from active sera has been recommended by Rinderknecht *et al.* [1939]. One would expect such strongly acid solutions to cause not only a decrease in activity but also some irreversible splitting of the serum proteins, and in our hands both these expectations have been realized. Severinghaus *et al.* [1938] have obtained promising preliminary results with the ultracentrifuge applied to pregnant mare's serum.

A study of the foregoing publications shows that a method is still awaited which will achieve an initial concentration of the gonadotropic hormone, providing, without serious loss, a stable, solid substance of moderate activity but sufficiently refined to be easily soluble and suitable for clinical use. Such a concentrate can be obtained by the method described below in a yield representing on the average 73 % of the initial activity of the serum. The method is economical and easily operated on a large scale. It is proposed to use the concentrate so obtained for studies in further purification, and considerable progress has already been made in this direction.

Methods

Assays. All activities have been determined, in comparison with the International Standard, by the method described by Day & Rowlands [1940], using ten rats for each assay, and the results are expressed in International Units (I.U.).

Protein determinations have been calculated from the total nitrogen (micro-Kjeldahl) or determined by the colorimetric method of Greenberg [1929] using Folin's phenol reagent, a tyrosine standard and an empirically determined factor of 22·8 [see Rimington & van den Ende, 1940].

Bound carbohydrate has been determined by the orcinol method as described by Rimington [1940].

p*H values* have been determined electrometrically using the quinhydrone electrode or, where insufficient material was available, by indicators, using buffer solutions for comparison.

Experimental

Eight pregnant mares were available for this work and from each, after preliminary tests to confirm pregnancy, about six bleedings were made (see Table 3), each of approximately 2 l. of blood, the intervals between successive bleedings being a week and being so arranged as to cover the period during which gonadotrophic activity of the serum rises to its maximum [Day & Rowlands, 1940]. Although mating was not observed, the mares and stallion being pastured together, the estimated dates, based upon Day's [1940] method of rectal palpation for the diagnosis of pregnancy, are accurate to ± 5 days. The blood was drawn into sterile receptacles containing 22 ml. of 10 % potassium oxalate per 2 l. of blood, the cellular elements were removed on the centrifuge and the resulting plasma clotted by addition, with vigorous agitation, of 3 ml. of 4 % calcium chloride to each 100 ml. of plasma. After straining, the serum was passed through a Seitz filter.

For kindly performing all these operations we are greatly indebted to Mr F. T. Day of the School of Agriculture, Cambridge.

After a small sample of each serum had been removed for biological assay, the remainder was stored at $-10°$ until required for chemical work.

Exploratory experiments with ammonium sulphate

The possibility of fractionation by means of ammonium sulphate was investigated by separating from 100 ml. of serum, diluted with an equal volume of water, the globulin fraction at 50 % sat. (further differentiated by dialysis into eu- and pseudo-globulin), crystalbumin, albumin I (50–66 % sat.), albumin II (66–75 % sat.) and albumin III (75–100 % sat.). Each fraction was dialysed and the protein, bound carbohydrate and gonadotrophic activity determined with the results shown for a typical experiment in Table 1.

Table 1. *Fractionation of active serum by* $(NH_4)_2SO_4$

Fraction	Activity		Carbohydrate
	% of total	I.U./mg. protein	Total N
Whole serum	100	1·58	0·212
Euglobulin	7·5	2·56	0·029
Pseudoglobulin	45	1·14	0·030
Crystalbumin	18	1·80	0·100
Albumin I	33·5	6·08	0·329
,, II	0	0	0·538
,, III	0	0	1·370

There is no sharp separation of activity in any one fraction. Albumin I possesses the greatest potency, but this fraction represents, in all, only one-third of the total activity. Precipitation of the active material is complete at 66 % sat. with $(NH_4)_2SO_4$, a fact confirmed in experiments upon purer material, although the albumins II and III are relatively more rich in bound carbohydrate.

Acidic protein precipitants

A sharp separation of gonadotrophic activity from inert material follows the use of salicylsulphonic acid, trichloroacetic acid or metaphosphoric acid as a deproteinizing agent. Rinderknecht *et al.* [1939] recommended adding a 33·3 % solution of salicylsulphonic acid to the serum until the *p*H lay between 1·9 and 2·2, and claimed a recovery of nearly 100 % of the activity in the filtrate. This is surprising in view of the known instability of serum gonadotrophin in acid solution, and in our hands yields of less than 60 % were obtained. We found, moreover, that under such drastic conditions some disintegration of the serum protein occurred, and the precipitate was consequently difficult to centrifuge, while the supernatant liquids exhibited a milk-like opalescence.

Trichloroacetic acid produced fairly complete deproteinization, but a high concentration of the reagent was required, with a correspondingly high acidity and rapid destruction of activity. A large proportion of the activity was found, further, to be carried down with the precipitate.

An ideal protein precipitant for the present purpose should operate in low concentration and without producing a high resultant acidity; it should also, if possible, combine with the protein so that any free excess is reduced to the minimum. These conditions are fulfilled by metaphosphoric acid, which we consider to be a reagent worthy of much more attention for the purpose of deproteinizing tissue fluids or extracts, as an initial stage in the isolation of comparatively labile chemical substances.

The protein-metaphosphoric acid reaction has been studied recently by Perlmann & Herrmann [1938] and by Briggs [1940]. The former authors pointed out that a salt-like combination occurs between the reactants and considered that the PO_3 bound reached a maximum when the $PO_3 : NH_2$ ratio was 1; the protein is not denatured by the reagent. Briggs confirms the statement that metaphosphoric acid reacts with the basic groups of proteins to form salt-like combinations with very low dissociation tendency, but he finds the relationship between the quantity added, as Na metaphosphate, to the system and that bound by the protein to follow a Freundlich adsorption isotherm, so that the ratio $PO_3 : NH_2$ in the flocculate increases continuously with increase in metaphosphate. No similar reaction occurs between metaphosphate and amino-acids or other substances of low molecular weight, containing single basic groups.

Optimal conditions for the HPO_3 precipitation

The relations between the quantity of metaphosphoric acid added, the proportion of protein precipitated, the final *p*H of the solution, and the recovery of activity in precipitate and supernatant respectively, were explored as follows. To 10 ml. lots of an active serum diluted 1 : 3 with distilled water were added differing quantities of a freshly prepared 0·3*N* HPO_3 solution. We have used throughout this work 'metaphosphoric acid, glacial in sticks' of British Drug Houses Ltd, and have found it convenient to work with the concentrations indicated. The addition was made dropwise to the well-stirred, ice-cold liquid; the precipitates were collected on the centrifuge, redissolved in water with the help of dilute alkali and made up to 10 ml. The *p*H of the supernatant fluids was determined on portions electrometrically, and on the remainders of these, and the redissolved precipitates, biological assays and determinations of protein (by phenol reagent) were performed. The results are shown in Fig. 1. At *p*H 3·5 the proportion of the total protein which is precipitated is 84 % and at *p*H 3·2 it has risen to 93·6 % with a corresponding improvement in the potency (activity per

mg.) of the unprecipitated active fraction; on the other hand, at reactions more acid than *p*H 3·5 loss of hormone from the filtrate becomes appreciable. While, therefore, separation of inert protein is not completed at *p*H 3·5, this end-point has been chosen as that giving the best all-round results for the reaction. The quantity of HPO_3 required to reach this end-point varies slightly, in accordance, no doubt, with the total protein content of the sera, but averages 475 ml. of the 0·3 *N* solution per l. of serum. When working up large batches of material, the

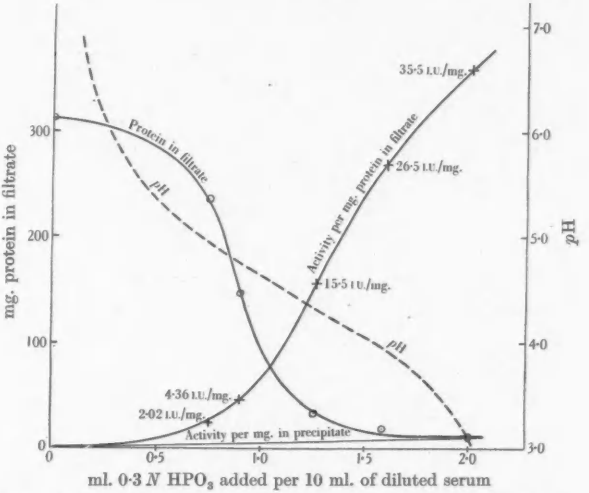

Fig. 1. Relations in the system: Pregnant mare's serum—metaphosphoric acid.

precipitated protein metaphosphate has been removed by passing the mixture through a Sharples super-centrifuge and the cake has been washed by resuspending it in 0·01 *N* HPO_3, using about 350–400 ml./l. of original serum, and again separating in the super-centrifuge. The combined filtrate and washings are neutralized by adding 20 % NaOH drop by drop with vigorous mechanical stirring.

The average recovery at this stage has been 95 % of the original activity.

Adsorption of the active material upon benzoic acid

Katzman & Doisy [1934] were able to adsorb gonadotrophic hormone from pregnancy urine by adding to it a saturated solution of benzoic acid in acetone, whereupon benzoic acid was precipitated and could be filtered off, carrying a considerable proportion of the activity with it.

We endeavoured to achieve a similar adsorption from our metaphosphoric acid filtrates, but the yields were disappointing; not only was adsorption incomplete and variable, but there was also appreciable inactivation of gonadotrophic hormone. Benzoic acid suspensions have a *p*H of about 3, and we suspect that destruction is a result of this comparatively high acidity. Much better results were obtained by liberating benzoic acid in the solutions by adding a mineral acid to sodium benzoate. The system benzoic acid : sodium benzoate possesses buffering power, and a closer study showed that we could precipitate a sufficient amount of the free acid in a finely divided form in our solutions, both

effectively and economically, without allowing the *p*H to fall below 4·5, a point at which there is little fear of inactivation of the hormone. Trials were made by using differing quantities of adsorbent at the same final *p*H, and also by varying the *p*H when the amount of benzoic acid in suspension was kept unchanged, from which it was found that adsorption takes place efficiently, from filtrates of widely varying activities, when the concentration of benzoic acid is about 5% and the final *p*H is 4·5–5·0 but not greater than 5·5. These conditions are reached in practice by adding, to the filtrates, 60 g. sodium benzoate per l. (equivalent to 5% benzoic acid) and then running, drop by drop, into the ice-cold, mechanically stirred solution, rather more than three-fourths of the equivalent quantity of 3*N* HCl. The final *p*H may be checked electrometrically or colorimetrically.

When working with large batches, the resulting precipitate has been collected by passing the mixture through a Sharples super-centrifuge, and unprecipitated benzoic acid has been recovered from the supernatant fluid, for purification and re-use, by addition of an excess of mineral acid.

We have omitted any aqueous washing of the benzoic acid with its adsorbed active material. The precipitate was scraped out and stirred with a quantity of acetone approximately equal to the volume of serum originally employed. This dissolves the benzoic acid and leaves the adsorbate as a finely flocculent material, which can be collected on the centrifuge, washed free from benzoic acid and moisture with three successive quantities of anhydrous acetone and one of dry ether, and then dried *in vacuo*.

Properties of 'initial powder'

The material obtained at this stage is a snow-white, light powder which dissolves slowly in water or buffer solution at *p*H 7·0–7·5, forming opalescent solutions which give positive biuret and Molisch tests, but contain no heat-coagulable protein. We have not been able to detect any change in the activity of samples kept during 8 months in stoppered bottles at room temperature. The yield of this material is about 6 g./l. of serum, and the activity ranges from 10 to 50 I.U./mg. It will be seen that only two simple chemical operations are necessary to obtain this comparatively stable product from active mares' serum which normally deteriorates unless stored under special conditions.

Factors affecting yields

From thirty-nine different sera, the activities of which varied over a wide range, we have been able to recover on the average 79% of the original activity in the form of the stable initial powder. Details of the volumes, activities, yields etc. at each stage are presented in Table 2, and the mean data for each individual mare are summarized in Table 3. Our conclusions may be summarized as follows:

(1) *Volume of serum and weight of initial powder.* There is a fairly constant relationship between these quantities, irrespective of the activity of the serum employed, the mean yield being approximately 6 g./l. As will be seen later, we believe that this powder is made up, for the greater part, of some constituent, probably glycoprotein in nature (see p. 746), which carries the gonadotrophic hormone with it.

(2) *Activity of serum and activity of initial powder.* If the entrainment of gonadotrophically active substance by the inert material accompanying it in the initial powder were complete, or always in the same proportion, a straight-line relationship would be expected between the potencies of powder and serum.

Table 2. *Data on gonadotrophic activity and recovery up to stage of 'initial powder' from thirty-nine sera*

Mare and no. of sample	Stage of pregnancy days	Volume of serum ml.	Activity of serum I.U./ml.	HPO_3 filtrate Recovery of activity* %	Initial powder		
					Weight g.	Activity I.U./mg.	Recovery of activity* %
J 6	44	665	270	119	4·464	42	106
7	51	1375	390	86	†4·207	40	65
					‡4·647	38	
8	58	1150	510	75	†3·677	56	72
					‡3·936	54	
9	65	1135	450	105	8·838	40	91
10	72	1080	300	102	7·072	42	92
L 3	45	975	100	91	6·302	20	110
4	53	1190	90	113	7·084	14	93
5	58	1980	210	77	12·113	24	70
6	67	850	180	93	5·846	30	116
7	74	1590	180	75	10·738	20	87
8	81	1355	120	89	7·817	20	98
M 3	49	940	170	102	5·084	26	84
4	56	1065	280	91	5·442	28	51
5	63	1320	360	96	12·179	20	54
6	70	1080	360	80	6·235	30	48
7	77	960	290	114	6·251	32	74
8	84	1090	230	78	8·211	26	88
N 3	53	1055	150	100	5·152	30	98
4	60	1042	330	105	6·267	36	66
5	67	1430	390	84	†3·877	57	79
					‡4·227	52	
6	74	1250	300	116	†3·941	38	81
					‡4·246	36	
7	81	900	210	106	5·069	30	81
8	88	1275	72	90	8·788	9	87
P 3	45	1290	140	78	6·745	15	64
4	53	1070	220	100	6·758	28	82
5	58	2340	420	116	13·285	28	41
6	67	1240	450	111	9·236	24	40
7	74	1130	240	113	7·117	26	69
8	81	1215	255	85	6·876	29	65
Q 5	80	1040	100	74	6·632	7	44
5A	80	620	64	—	3·230	11	90
R 2	59	1940	145	117	8·803	30	95
3	65	2170	155	106	12·348	24	88
S 4	47	817	145	101	3·484	34	102
5	54	1165	180	92	4·093	40	79
6	61	1390	240	94	7·683	34	78
7	68	1435	190	101	7·539	38	105
8	75	1220	145	92	6·324	18	65
9	82	1250	65	83	6·347	8	62

* Allowance has been made for samples withdrawn from the diluted serum for assay.
† 5% benzoic acid suspension. ‡ 10% benzoic acid suspension.

Fig. 2 demonstrates that this expectation is not fully realized. With sera containing up to 250 I.U./ml., the activity of the powder is very approximately a linear function of the activity of the serum, but with more highly active sera there is a progressive deviation from this ideal relationship. The experimental data can be more readily interpreted if the process of concentration of the activity be regarded as one of adsorption or, more strictly, of co-adsorption; gonadotrophin being first adsorbed by the inert serum constituent and this latter, with its attached active material, becoming secondarily adsorbed upon the benzoic acid precipitate liberated in the system. The latter adsorbent must be

Table 3. *Summary of data for individual mares*

No. of mare	No. of bleedings	Volume of serum extracted l.	Mean activity of serum I.U./ml.	Recovery in HPO_3 filtrate %	Initial powder: Total weight g.	Initial powder: Mean activity I.U./mg.	Initial powder: Recovery %
J	5	5·405	385	97	36·841	45	85
L	6	7·940	147	90	49·900	21	96
M	6	6·455	282	94	43·400	27	67
N	6	6·952	242	100	41·585	36	82
P	6	8·285	287	101	50·020	25	60
Q	2	1·660	82	74	3·394	9	67
R	2	4·110	150	112	21·151	27	91
S	6	7·277	161	94	35·469	29	82
	39	48·084		95	281·760	27	79

present, however, in such quantity that adsorption upon it is virtually complete. The factor limiting the final yield of gonadotrophin would then be the relative quantity of the inert constituent supplied by the serum; increasing the amount of benzoic acid used should have little, if any, effect. This is borne out by the

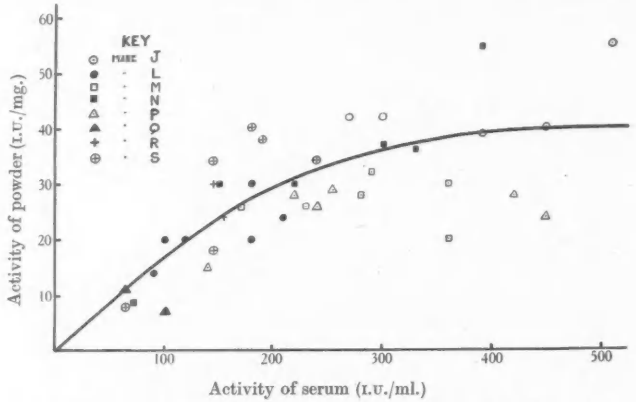

Fig. 2. Relation between activity of serum and potency of initial powder.

results of experiments in which the HPO_3 filtrates from highly active sera were divided, and one-half worked up as usual with 5% of benzoic acid, the other half with twice this amount—see Table 4.

Table 4. *Effect of concentration of benzoic acid on recovery of gonadotrophin from highly active sera*

Serum	Activity I.U./ml.	Conc. of benzoic acid %	Wt. of powder g.	Activity I.U./mg.	Recovery of activity %
J 7	390	5	4·207	40	63
		10	4·647	38	66
J 8	510	5	3·677	56	70
		10	3·936	54	72
N 5	390	5	3·877	57	79
		10	4·227	52	79
N 6	300	5	3·941	38	80
		10	4·264	36	82

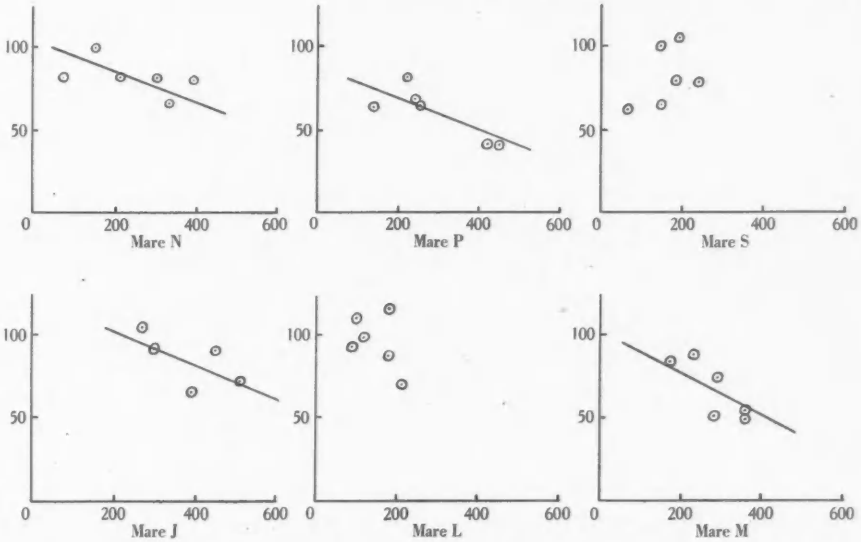

Fig. 3. Relation between activity of serum and percentage recovery in initial powder from individual mares. Abscissa = I.U./ml. of serum. Ordinate = percentage recovery.

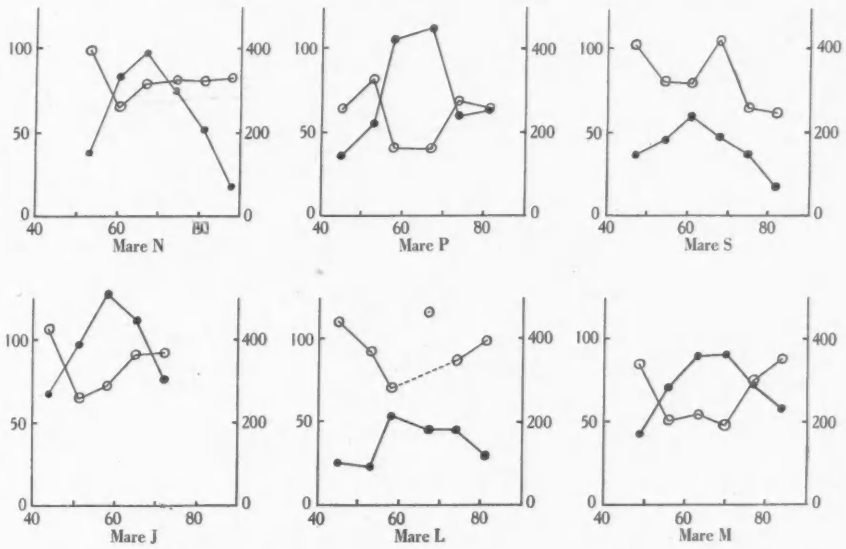

Fig. 4. Relation between activity of serum •—•—•, percentage recovery in initial powder o—o—o and stage of pregnancy for individual mares. Abscissa = stage of pregnancy in days. Ordinates = left: percentage recovery; right: I.U./ml. of serum.

(3) *Activity of serum and percentage recovery in initial powder.* Fig. 3 shows for each mare separately (omitting Q and R from which two bleedings only were obtained) the relationship between the original activity of the serum samples and the percentage of this activity finally recovered in the form of the 'initial powder'. In four out of six mares there is a regular fall in percentage recovery as the activity of the serum increases; in the remaining two cases, mares S and L, the peak activity usually encountered about the 65th day of pregnancy was much less marked, and the bleedings, therefore, provide insufficient evidence for such a comparison. These relationships are also brought out in Fig. 4, which relates stage of pregnancy to activity per ml. of serum and percentage recovery of activity.

The choice of a highly active serum as starting material for the preparation of gonadotrophic concentrate is not without its drawbacks. By pooling sera of higher and lesser activities, the optimal conditions for obtaining maximum recovery are likely to be more nearly approached.

A second feature discoverable from Fig. 3 is the difference between individual mares, some yielding sera from which a consistently better percentage yield of activity was obtained than others, e.g. the mean yield from the five bleedings of mare J was 85% and that from the six bleedings of mare P only 60% of the original activity of the sera. Such individual differences may, possibly, be due to differences in the proportion of 'inert protein adsorbent' in the sera.

Further concentration by fractional alcohol precipitation

The crude initial powder resulting from the benzoic acid adsorption can be further purified by alcohol precipitation at suitable *p*H levels; in particular, the sparingly soluble impurities can be eliminated and a material obtained assaying up to 625 I.U./mg. At this stage of the process we have been guided by the experience of Gurin *et al.* [1939] with urinary gonadotrophin, but we have modified their technique by adding alcohol to the neutral solution and then titrating with alcoholic acetic acid to *p*H 4·8, instead of shaking the powder directly with buffered alcohol. By this simplification, one complete set of manipulations, the extraction with 50% alcohol at *p*H 6·0, is eliminated; moreover, by titrating instead of washing repeatedly with solvent a great saving of time is effected and, in our experience, higher yields are obtained. The method may be described as follows.

The initial powder is brought into solution by grinding it with forty times its weight of water containing 4·4 ml. 0·1 *N* NaOH per g. of powder, which is sufficient to bring the *p*H to about 7·4. It is advisable to stir the resulting mixture mechanically for some hours and to leave it standing in the ice chest overnight. An equal volume of alcohol ('Methcol') is then added and, while stirring mechanically, a 5% solution of acetic acid in 50% alcohol is added drop by drop until *p*H 4·8 is reached;[1] this requires approximately 1·1 ml./g. of initial powder.

The heavy precipitate which is formed is collected on the centrifuge after chilling the mixture for 1–2 hr., and washed three times by stirring with 50% alcohol buffered at *p*H 4·8 (60 ml. *N* CH_3COONa + 40 ml. *N* acetic acid + 100 ml. water + 200 ml. alcohol) and recentrifuging. To the combined supernatant fluids an equal volume of alcohol is added (to 75%), whereupon a fine flocculent precipitate is formed. The mixture is left overnight at 0° and the precipitate then

[1] A small drop of the solution is mixed with a drop of water on a glazed plate; bromocresol green is added and the colour compared with that given by a similarly diluted drop of 50% alcohol containing acetate buffer at *p*H 4·8. Values so obtained have been checked by the quinhydrone electrode.

collected on the centrifuge, washed once with each of the following: 80 % alcohol, absolute alcohol, acetone and dry ether; it is finally dried *in vacuo*.

We have found the yields by our method to be very good, approximately 92 % by weight of the initial powder being removed, leaving, on the average, 92 % of the activity in the concentrate (see Table 5).

Table 5. *Purification of initial powder by 50 % alcohol at* p*H 4·8*

	Initial powder		Concentrate		
No.	Wt. g.	Activity I.U./mg.	Wt. g.	Activity I.U./mg.	Recovery of activity %
L 5 (8)	0·189	36·4	0·0446	148	95·9
N 8 (1)	7·18	10	0·362	190	94·4
N 7 (1)	5·06	30	0·414	320	87·3
PMS 33 (1)	19·36	50	1·639	625	105·1
PMS 30	7·83	26	0·619	311	94·5
PMS 32	14·35	33·5	1·272	287	76·1
					Mean 92·2

During large-scale operations, a further economy might possibly be introduced by reworking the alcohol-insoluble residues for the modicum of activity they retain. As an example, the fraction of PMS 33 (1) insoluble in 50 % alcohol (see Table 5) and containing 5·3 I.U./mg. was redissolved in dilute alkali and the process of alcohol fractionation repeated. This material, which we designated PMS 33 (2), thus yielded a fraction soluble in 50 % alcohol, representing 81·3 % of the total activity and having a potency of 227 I.U./mg., while the residue assayed only 1·4 I.U./mg.

Properties of the product obtained after alcohol precipitation

The material obtained from the fraction soluble in 50 % alcohol at *p*H 4·8, but insoluble at 75 % concentration, is a white powder, very readily and completely soluble in water; solutions give positive biuret and glyoxylic tests. The gonadotrophic activity of our preparations has ranged from 150 to 625 I.U./mg., and there appears to be a correlation between the activities of the concentrates and those of the initial powders from which they were derived (see Table 5). The histological response elicited in the ovaries of hypophysectomized rats by the injection of the purified product is similar to that given by untreated serum.

Carbohydrate content of fractions prepared by 50 % alcohol

Determinations of carbohydrate content have been made at each of the principal stages in the concentration of serum gonadotrophin, but until the stage of alcohol fractionation was reached no reliable information could be gained concerning the nature of the hormone, owing to the large proportion of impurity still present. It was noted, however, that the initial powders afforded the relatively high figure of 7–8 % of bound hexose, resembling, in this respect, seromucoid and other typical glycoproteins.

The carbohydrate contents have now been determined of the 50 % alcohol concentrates in five out of the six samples listed in Table 5, and also in their corresponding fractions insoluble in 50 % alcohol at *p*H 4·8. The results show a striking difference between the two materials, the relatively inert fractions containing about 8 % of bound hexose and the active fractions from 2·5 to 3·5 % (see Table 6).

Table 6. *Carbohydrate content of fractions separated by 50% alcohol at pH 4·8*

	Insoluble fraction		Soluble fraction	
No.	Activity I.U./mg.	Hexose %	Activity I.U./mg.	Hexose %
N 8 (1)	0·7	8·32	190	2·94
N 7 (1)	2·7	8·42	320	2·54
PMS 33 (1)	5·3	7·40	625	3·44
PMS 33 (2)	1·4	7·80	227	2·41
PMS 30	1·9	8·38	311	2·84
PMS 32	1·9	7·78	287	3·87

This result is somewhat surprising, in view of the suggestion of Li *et al.* [1940] that their highly purified gonadotrophic extract from pregnant mare's serum is a glycoprotein, and the figure of 14·1% hexose quoted in respect of it by them. Their statement that 'the carbohydrate determination may be taken as a measure of PMS potency in the purification of the hormone' is clearly invalid. Our preparations of about 600 I.U./mg. undoubtedly still contain much impurity, but carbohydrate content and gonadotrophic activity are, at this stage, running in opposite directions. An interesting illustration is afforded by sample PMS 33 (2). This was the alcohol-insoluble fraction of the large batch PMS 33 (1), and was refractionated by the same process (see above). The active fraction obtained (2% of the weight) contained 81·3% of the activity and 2·4% hexose, while the insoluble fraction, containing 23·1% activity, had a hexose content of 7·8%, slightly higher than the initial figure of 7·4% before fractionation.

Discussion

The method most usually adopted for the preparation of active concentrates from pregnant mare's serum is that of Cartland & Nelson as modified by Goss & Cole [1940]. The latter authors state, however, that the yield from the initial acetone precipitation is less than 60% of the original activity of the serum, and for their final products no yields are quoted. The method employs large volumes of acetone which would be difficult to recover, since they contain the water, salts etc. of the serum, and large vessels are required to handle the big bulk of fluids.

The method described in the present paper is simple and comparatively economical; the acetone and benzoic acid used can be recovered with ease, thus lessening working costs. To work up 100 l. of serum we calculate the requirements of the two methods as follows:

Cartland and Nelson's method	*Method here described*
500 l. acetone (not easily recoverable)	1·4 kg. metaphosphoric acid
Smaller quantities of alcohol, acids, etc.	20 kg. benzoic acid } Recoverable Approx. 100 l. acetone } for re-use
	Smaller quantities of alcohol, acids, etc.

By our method, a material is obtained representing 73% of the original activity of the serum and with an activity of 150–625 I.U./mg. For therapeutic use it may be mixed with a suitable proportion of an inert diluent.

Investigation is proceeding upon the further purification of the hormone with a view to its ultimate isolation and chemical characterization.

Protocol

As an example of the working of the method, a single preparation will be described in detail.

Serum N 7 from mare N when 81 days in foal; volume 900 ml.; activity assayed at time of preparation 210 I.U./ml.

To 900 ml. of serum were added 1800 ml. of water, and 20 ml. were removed for assay. The remainder was surrounded by ice and mechanically stirred while 430 ml. of 0·3 *N* HPO_3 were added drop by drop at the rate of about 1 drop/sec. The *p*H, determined electrometrically, was then 3·3. The mixture was passed through a Sharples super-centrifuge and the cake of protein metaphosphate resuspended by grinding in 1 l. of 0·01 *N* HPO_3. This mixture was passed through the machine and the clear fluid added to the main filtrate, which was neutralized to *p*H 7·0 by addition, with mechanical stirring, of sufficient 20% NaOH. The volume of filtrate was 4140 ml. 20 ml. were removed for assay. A solution of sodium benzoate was prepared by boiling 207 g. benzoic acid in 200 ml. water and 136 ml. of 50% NaOH, and cooling the mixture to room temperature.

The HPO_3 filtrate was surrounded by ice and mechanically stirred; the sodium benzoate solution was added and then 3 *N* HCl was allowed to drop into the briskly stirred mixture at a rate not greater than 1 drop per sec. 450 ml. of acid were required to bring the *p*H to 4·55. The mixture was put through the Sharples super-centrifuge and the cake of benzoic acid scraped out and stirred with about 1 l. of acetone.

When all the lumps had disintegrated, the fine precipitate was allowed to settle and the bulk of the supernatant acetone removed. The precipitate from the remainder was then collected on the centrifuge (using 200 ml. capacity glass bottles), washed three times on the centrifuge with about 400 ml. in all of anhydrous acetone and finally once with about 150 ml. of anhydrous ether. The last traces of ether were removed from the 'initial powder' by exposing it over sulphuric acid in a vacuum desiccator overnight.

Weight of powder recovered 5·069 g.
Activity 30 I.U./mg.
Recovery of original activity of serum 81%.

5·06 g. of initial powder were ground up with 200 ml. of water containing 2·2 ml. of *N* NaOH and the opalescent solution which had *p*H 7·4, was stirred mechanically for 2 hr. and then left in the ice chest overnight. 200 ml. of alcohol were added and then dropwise, while stirring, 7 ml. of 5% acetic acid in 50% alcohol, which brought the *p*H to 4·7. After chilling for 1·5 hr. the mixture was centrifuged and the precipitate washed on the centrifuge three times with 50% alcohol at *p*H 4·8 (see p. 744). To the combined supernatant fluids an equal volume of alcohol was added and the mixture left at 0° overnight. The small precipitate was collected on the centrifuge, washed once with 80% alcohol, absolute alcohol, acetone and dry ether, then freed from solvent *in vacuo* over sulphuric acid.

Weight of powder recovered 0·4141 g.
Activity 320 I.U./mg.
Recovery of activity 87·3%.
Overall recovery from serum 71%.

The material insoluble in 50% alcohol at *p*H 4·8 was dehydrated with acetone and ether. It weighed 4·475 g. and contained 2·7 I.U./mg., accounting for 8% of the total activity.

Summary

A method is described for the preparation from the serum of pregnant mares of a stable, dry concentrate. The method is economical and is suitable for large-scale operation and depends firstly upon precipitation of inert serum proteins by metaphosphoric acid. Very little loss in gonadotrophic activity occurs at this stage. The active principle is then adsorbed upon benzoic acid and by solution of the latter in acetone a stable initial powder is obtained averaging 27 I.U./mg.

and accounting for 79% of the activity (data compiled from thirty-nine individual preparations totalling 48 l. of serum).

The initial powder is further fractionated by means of titration in 50% alcohol to *p*H 4·8; an insoluble portion representing 92% by weight and practically devoid of activity is rejected, while from the solution the active fraction is recovered by further addition of alcohol. This material contains 150–625 I.U./mg. and is readily soluble in water. The average yield at this stage is 92%, making an overall yield of 73% of activity from the original serum.

The chemical properties of the active material have been investigated: it contains 2·5–3·5% of hexose (orcinol method), while the inert fraction insoluble in 50% alcohol at *p*H 4·8 contains 7·5–8·5% of hexose.

Investigation is proceeding upon the further purification of the hormone.

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